The Molecular Mechanisms Mediating Dendritic Cell Responses to Anti-inflammatory Microbes

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2. Summary

The microbiota in the gastrointestinal tract influence immune homeostasis. Many microbes have different effects on the immune system. Enterogenic pathogens induce strong inflammatory responses within the gastrointestinal tract, while commensal bacteria induce tolerance. One of the intestinal tolerogenic bacteria is *Bifidobacterium infantis* 35624 (*B. infantis*), which has been previously shown to protect against inflammatory diseases in murine models, such as colitis and respiratory allergy. The protective host immune response was demonstrated to include the induction of T regulatory cells. However, the molecular basis for this effect was not well understood.

In this thesis the molecular mechanism responsible for *B. infantis* recognition by human monocyte-derived dendritic cells (MDDCs), myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) was investigated. Moreover, the influence of dendritic cell (DC) subsets on naïve T cell polarization was assessed. *B. infantis* stimulated MDDCs, mDCs and pDCs secreted interleukin (IL)-10 with low levels of IL-12p70 and expressed indoleamine 2,3-dioxygenase (IDO). This microbe did not induce interferon (IFN)-α in pDCs. MDDCs and mDCs IL-10 secretion was Toll-like receptor (TLR)2/6 dependent, while pDCs IL-10 secretion was TLR9 dependent. Moreover, MDDCs and mDCs expressed retinaldehyde dehydrogenase 2 (RALDH2) in response to *B. infantis*. RALDH2 expression was TLR2 and DC-SIGN dependent. Co-culture of naïve T cells with bacteria primed DC subsets resulted in enhanced expression of Foxp3 in CD4+CD25+ cells. Foxp3+ T cell induction by MDDC and mDC required TLR2, DC-SIGN and retinoic acid, while for pDCs IDO was required. Enhanced expression of Foxp3 in T lymphocytes was also observed in peripheral blood of healthy volunteers, who received *B. infantis* for 8 weeks. Moreover, these T regulatory (Treg) cells displayed strong regulatory phenotype, including inducible T-cell costimulator (ICOS) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression.

As *B. infantis* enhanced RALDH2 expression and retinoic acid production by MDDCs and mDCs, the importance of this metabolite was further examined in animal models. Retinoic acid metabolizm by mucosal $CD103^+$ DCs after *B. infantis* exposure was confirmed and the role of *B. infantis*- induced retinoic acid metabolism in protection against dextran sodium sulphate (DSS)-induced colitis was analyzed.

B. infantis feeding led to increased number of $CD103^+RALDH^+$ DCs in the small intestinal lamina propria (LP) of healthy mice. $Foxp3^+$ Treg cells were also elevated in LP, while T helper (Th)1 and Th17 subsets were decreased. Citral (RALDH enzyme inhibitor) treatment blocked the increase in $CD103^+RALDH^+$ DCs and the decrease in Th1 and Th17 subsets. There was no citral effect on the $Foxp3^+$ Treg cell population, which suggests that other immunoregulatory factors may play a role. *B. infantis* reduced the severity of DSS-induced colitis. This effect was associated with decreased Th1 and Th17 cells in LP. Moreover, in the colon there was less neutrophil infiltration, measured using myeloperoxidase activity. Citral treatment confirmed that these effects were related to retinoic acid metabolism. $CD11b^+CD103^+$ DCs increased and $RALDH^+$ DCs decreased in LP of control colitic mice. In contrast, numbers of $RALDH^+$ DCs in LP of *B. infantis*-fed animals were maintained.

In this thesis two different mechanisms of Foxp3+ T cells induction by *B. infantis* activated DCs are presented: (1) TLR2, DC-SIGN and retinoic acid-dependent Treg induction by mDCs; (2) IDO-dependent Treg induction by pDCs. RALDH2 upregulation and retinoic acid involvement were specific for this microbe. Two related *Bifidobacteria* strains *B. animalis* and *B. globosum* were not able to induce retinoic acid metabolism in DCs, suggesting that not all microbes have the same immunomodulatory properties. Thus, $CD103^+$ small intestine LP DCs producing retinoic acid are a potential cellular target for microbiota-mediated effects on mucosal immunoregulation and homeostasis. Cross-talk between many pattern recognition receptors and metabolic pathways determines the response to *B. infantis*. RALDH2 expression and retinoic acid production link nutrition (vitamin A), commensal microbiota and tolerance induction. These results suggest that supplementation with specific tolerogenic microbes may provide benefits to patients suffering from wide range of mucosal inflammatory disorders.

3. Zusammenfassung

Die mikrobielle Besiedelung des Magen-Darm-Trakts hat einen Einfluss auf die Homöostase des Immunsystems. Nicht alle Mikroben haben denselben Effekt. So verursachen enterogene Pathogene eine starke Entzündungsantwort im Gegensatz zu kommensalen Bakterien, die Toleranz induzieren. *Bifidobacterium infantis* 35624 *(B. infantis)* ist so ein toleranzinduzierendes Darmbakterium, von dem schon bekannt war, dass es in Mausmodellen eine schützende Wirkung gegen die Entstehung von Entzündungskrankheiten wie Kolitis und Atemwegsallergien hat. Diese schützende Wirkung wurde der Aktivierung von regulatorischen T Zellen zugeschrieben. Nichtsdestotrotz sind die molekularen Grundlagen für diesen Effekt bislang nicht ausreichend verstanden.

In der vorliegenden Arbeit wurde der molekulare Mechanismus untersucht, wie *B. infantis* von humanen aus Monozyten differenzierten dendritischen Zellen (MDDC), myeloiden dendritischen Zellen (mDC) und plasmazytoide dentritische Zellen (pDC) erkannt wird. Zusätzlich wurde der Einfluss der dendritischen Zellsubtypen auf die Polarisierung naiver T Zellen untersucht. Wir fanden, dass mit *B. infantis* stimulierte MDDCs, mDCs und pDCs Interleukine (IL)-10 und ein wenig IL-12p70 sezernieren, zusätzlich exprimieren sie die Indoleamin 2,3-dioxygenase (IDO). Die Expression von Interferon (IFN)-α in pDCs wurde nicht beeinflusst. Die IL-10 Sekretion von MDDCs und mDCs ist abhängig von TLR2/TLR6 während diejenige von pDCs von TLR9 abhängig ist. Dann exprimierten MDDCs und mDCs RALDH2 als Antwort auf *B. infanits* Stimulation. Diese Expression war abhängig von TLR2 und DC-SIGN. Ko-Kulturen von naiven T Zellen mit dendritischen Zellen, welche mit den Bakterien voraktiviert wurden, erhöhten die Expression von Foxp3 in CD4+CD25+ Zellen. Die Induktion von Foxp3+ T Zellen mit MDDCs und mDCs benötigte TLR2, DC-SIGN und Retinolsäure, während bei pDCs IDO nötig war. Eine Erhöhung der Foxp3 Expression in T Lymphozyten wurde ausserdem in peripherem Blut von gesunden Freiwilligen gefunden, welche *B. infantis* während 8 Wochen konsumiert hatten. Der regulatorische Phänotyp dieser Zellen wurde durch die Expression vom induzierbarem T Zell Ko-Stimulator (ICOS) und vom Zytotoxischen T Lymphozyten Antigen 4 (CTLA-4) unterstrichen.

Weil *B. infantis* die RALDH2 Expression und somit die Retinolsäureproduktion in MDDCs und mDCs erhöht, untersuchten wir die Wichtigkeit dieses Prozesses in

Tiermodellen. So konnten wir die Retinolsäuremetabolisierung in CD103+ dendritischen Zellen der Schleimhaut nach Exposition gegenüber *B. infantis* bestätigen. Zusätzlich haben wir die Rolle der Retinolsäuremetabolisierung nach *B. infantis* Kontakt in einem Dextran Natriumsulfat (DSS)-induzierten Kolitismodell in Mäusen analysiert. Fütterung von *B. infantis* führte zu höheren Zahlen an CD103+RALDH+ dendritischen Zellen in den Lamina Propria (LP) Zellen des Dünndarms von gesunden Mäusen. Zusätzlich waren die Foxp3+ regulatorischen T Zellen erhöht in LP, während TH1 und TH17 Subtypen reduziert waren. Zusätzliche Behandlung der Mäuse mit Citral (ein RALDH Enzym Inhibitor) inhibierte die Erhöhung von CD103+RALDH+ dendritischen Zellen, die Reduktion der TH1 und TH17 Subtypen blieb aber bestehen. Wir beobachteten auch keinen Citral Effekt auf die Foxp3+ regulatorischen T Zellpopulation, woraus wir schliessen, dass zusätzlich andere immuno-regulatorische Faktoren eine Rolle spielen könnten. Dann reduzierte *B. infantis* die Symptome der DSS-induzierten Kolitis. Dieser Effekt war assoziiert mit weniger TH1 und TH17 Zellen in LPs. Zusätzlich war der Darm weniger von Neutrophilen Zellen infiltriert, was durch Messung der Myeloperoxidase Aktivität bestimmt wurde. Die Behandlung der Mäuse mit Citral bestätigte, dass diese Effekte im Zusammenhang mit der Retinolsäuremetabolisierung stehen. In den LP von unbehandelten Mäusen, die an Kolitis erkrankt waren, fanden wir mehr CD11b+CD103+ dendritische Zellen während die RALDH+ dendritischen Zellen reduziert waren. Im Gegensatz dazu waren die RALDH+ dendritischen Zellen durch die B. infantis Fütterung erhöht.

In der vorliegenden Arbeit stellen wir zwei verschiedene Mechanismen zur Foxp3+ T Zell Induktion vor: (1) mDCs, welche regulatorische T Zellen in Abhängigkeit von TLR2, DC-SIGN und Retinolsäure induzieren; (2) pDCs, welche regulatorische T Zellen IDO-abhängig induzieren. Die RALDH2 Hoch-Regulierung und die Retinolsäure Produktion ist spezifisch für *B. infantis*. Zwei Verwandte Bifidobakterienstämme B.animalis und B.globosum führten nicht zur Retinolsäuremetabolisierung in dendritischen Zellen. CD103+ dendritische Zellen der LPs des Dünndarms, welche Redinolsäure produzieren, wurden schon als potenzielles Ziel des Mikrobiota-vermittelten Effekts auf die Immunregulation und Homeostase der Schleimhäute beschrieben. Das Zusammenspiel verschiedener Mustererkennungsrezeptoren und metabolischer Wirkungswege bestimmt die Wirkung von B. infantis. Die Expression von RALDH2 und die Retinolsäureproduktion verbinden die Ernährung (Vitamin A), die Darmflora und die Toleranzinduktion des

Immunsystems. Unsere Resultate zeigen auf, dass die Ergänzung der Nahrung mit toleranzinduzierenden Mikroben Patienten mit Entzündungskrankheiten der Schleimhäute helfen könnte.

4. Abbreviations

ADH	aldehyde dehydrogenase
AIEC	adherent-invasive *E. coli*
APC	antigen presenting cell
ASC	antigen specific cell
B. infantis	*Bifidobacterium infantis* 35624
BmDC	bone marrow-derived dendritic cell
cAMP	cyclic adenosine monophosphate
CARD	caspase recruitment domain
CBP	CREB-binding protein
CCL	CC chemokine ligand
CCR9	CC chemokine receptor 9
CFSE	carboxyfluorescein succinimidyl ester
CLA	conjugated linoleic acid
CLR	C-type lectin receptor
CRABP	cellular retinoic acid binding protein
CRD	carbohydrate recognition domain
CREB	cAMP response element-binding protein
CRP	C reactive protein
CTLA-4	cytotoxic T-lymphocyte antigen 4
CX3CR1	CX3C chemokine receptor 1
DAMP	danger-associated molecular pattern
DC	dendritic cell
DCAR	dendritic cell immunoactivating receptor
DCIR	DC immunoreceptor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DSS	dextran sodium sulphate
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated kinases
FABP5	fatty acid binding protein 5
Foxp3	forkhead box P3
GABA	γ-amino butyric acid

GALT	gut-associated lymphoid tissue
GM-CSF	granulocyte macrophage colony-stimulating factor
GPCR	G protein coupled receptor
IBD	Inflammatory Bowel Disease
ICER	inducible cAMP early repressor
ICOS	inducible T-cell costimulator
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IPS-1	interferon promoter-stimulating factor 1
IRAK	IL-1 receptor-associated kinase
IRF	IFN-regulatory factor
iTreg	inducible T regulatory cell
JNK	c-Jun N-terminal kinase
LAG3	lymphocyte-activation gene 3
LBP	LPS-binding protein
LCFA	long-chain fatty acid
LN	lymph node
LP	lamina propria
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAdCAM	mucosal addressin cellular adhesion molecule
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated gene 5
mDC	myeloid dendritic cell
MDDC	monocyte-derived dendritic cell
MDP	muramyl dipeptide
MGL	macrophage galactose-type lectin
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MyD88	myeloid differentiation primary response protein 88

NLR	NOD-like receptor
NLRC	nuclear oligomerization domain proteins subfamily C
NOD	nonobese diabetic mice
NOD1	nucleotide-binding oligomerization domain 1
NOD2	nucleotide-binding oligomerization domain 2
PAMP	pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PGE2	prostaglandin E2
PLN	peripheral lymph node
PP	Peyer's patch
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PSA	polysaccharide A
PTGN	peptidoglycan
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol-binding protein
RDH10	retinol dehydrogenase 10
RIG-I	retinoic acid inducible gene I
RIP1	receptor-interacting protein 1
RLR	RIG-I-like receptor
RXR	retinoid X receptor
SCFA	short-chain fatty acid
SDR	short chain dehydrogenase reductase
SHP-1	Src homology phosphatase-1
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SOCS3	suppressor of cytokine signaling 3
STAT6	signal transducer and activator of transcription 6
TAK1	TGF-β-activated kinase 1
TGF-β	transforming growth factor beta
Th	T helper

TIR	Toll-interleukin 1 (IL-1) receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRADD	TNF receptor type 1-associated DEATH domain protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTR	transthyretin
VAD	vitamin A deficient mice

5. Introduction

5.1 Human gut microbiota

Our epithelial surfaces are colonized not only by numerous bacteria species, but a diverse microbial community, which consists of bacteria, archea, fungi, protozoa and viruses (1-5). Recent developments in high-throughput sequencing have uncovered a higher diversity in human microbiota than expected (6, 7). It was demonstrated that the microbiota encode at least 100-fold more specific genes than found within the human genome (8), which raises the question as to how big an impact they can have on the human host.

Greater than 10^{14} bacteria colonize the mucosal surfaces of the body. Especially rich in bacteria is the lower gastrointestinal tract. It harbors trillions of beneficial commensal bacteria, numbering at least 1000-5000 species (9). Most of them are from the phyla *Bacteroidetes* or *Firmicutes*, whereas *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Fusobacteria* are represented at lower levels (10-12). Moreover, the human gut is colonized by methanogens, which belong to *Archea* (13). Microbiota distribution throughout the gastrointestinal tract depends on many factors such as host-microbial, microbial-microbial interactions and nutrients accessibility. In healthy individuals, *Proteobacteria* spp (mainly *Enterobacteria*), *Lactobacillales* and *Erysipelotrichales* (especially *Turicibacter* spp) dominate in the small intestine. The large intestine is colonized mainly by *Bacteroidetes* and *Clostridia* (Figure 1) (14).

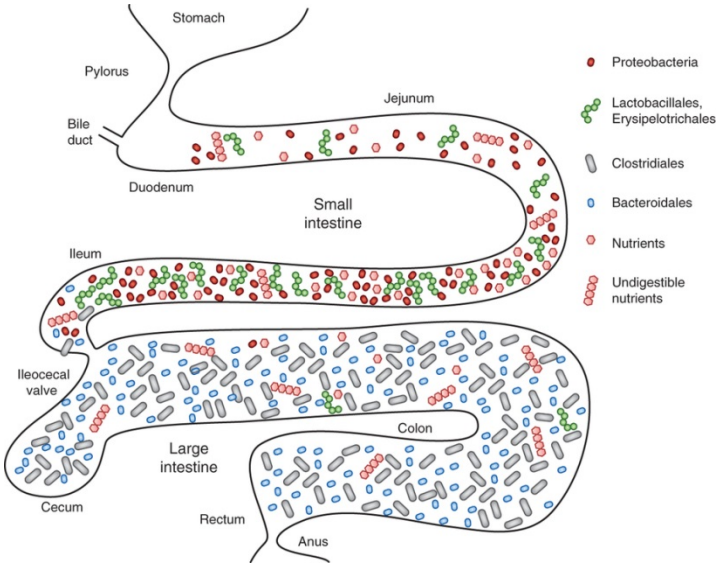

Figure 1. Dominant bacterial groups within the gastrointestinal tract. (Reproduced from Kamada N, Nat Immunol 2013) Small intestine, rich in easily accessible nutrients, is colonized by *Proteobacteria*, *Lactobacillales* and *Erysipelotrichales* (15). In contrast, large intestine contains undigestible nutrients, which facilitates growth of diverse *Bacteroidales* and *Clostridiales.*

These differences are strongly related to nutrient distribution. The small intestine is rich in monosaccharides, disaccharides and amino acids used by host and microbes, while the large intestine containes host-indigestible fibers. Small intestinal bacteria, such as *Proteobacteria*, cannot digest polysaccharides and cannot use them as an energy source. In contrast colon bacteria, such as *Bacteroidetes*, possess the ability to scavenge polysaccharides (14). Nevertheless, this feature can be highly diverse even for bacteria from the same phyla and partially explains why the colon is the most diverse in bacteria species within the gut. Some bacteria, such as methanogens, do not use sugars as an energy source, instead they obtain energy from hydrogen molecules, which are a side product of obligate anaerobic metabolism (13). The intestinal environment is also rich in ecological networks, which allows some commensals to acquire critical nutrients from their intestinal neighbors. For example, *Eubacterium dolichum* and *Lactobacillus johnsonii* cannot synthetize certain amino acids and need to incorporate them from the intestinal environment (16, 17). Changes in any of these components lead to alteration in the entire system, which was demonstrated following antibiotic use and diets based on fat and protein or vegetable fiber components.

There is no doubt that diet impacts intestinal microbiota composition from early life. The breast feeding of infants favors colonization by more *Bifidobacteria* species compared to formula fed infants (18, 19). Recently, association between diet and gut microbiota was investigated in human adults. Existence of three gut microbiota groups, called enterotypes, was proposed (20). They were dominated by *Bacteroides*, *Prevotella* and *Ruminococcus*. A diet high in fat and animal protein with low carbohydrate intake resulted in high levels of *Bacteroides* and low level of *Prevotella*. In contrast, diets high in simple carbohydrates and low in fat and animal protein showed an inverted pattern with low *Bacteroides* and high *Prevotella*. These results were observed only with long-term dietary habits. In the case of short-term diet changes, the gut microbiota has the ability to respond within 24hours, although it does not lead to an enterotype switch (21).

The microbiota in the gut lumen is continuously monitored by the gut-associated lymphoid tissue (GALT) (Figure 2). The monolayer of intestinal epithelial cells (enterocytes) forms a barrier between the luminal microbiome and lamina propria (LP) cells. It is worth to mention that bacteria can be already recognized by a limited number of receptors expressed on enterocyte surface. However, microbes are mainly sampled by LP dendritic cells (DCs) reaching their dendrites into the gut lumen, between tight junctions on enterocytes. Alternatively, microbes can be delivered to Peyer's patches (PP) through M cells, which are specialized epithelial cells in the intestinal epithelial cell lining. Subsequently, microbes are recognized and taken up by PP resident DCs. Bacterial components are recognized via diverse pattern recognition receptors (PRRs), which are present on the surface, in the intracellular compartments and in the cytoplasm of DCs. Moreover, DCs respond to microbial metabolites produced in the gut lumen during fermentation processes. Bacterial activated DCs secrete diverse cytokines, immunomodulatory factors and interact with T and B lymphocytes, which leads to pro- or anti-inflammatory adaptive responses. Antigen presentation takes place locally in LP and after DC migration to the mesenteric lymph node (MLN). In addition, some DCs can stay within PP and there prime naïve T cells (22).

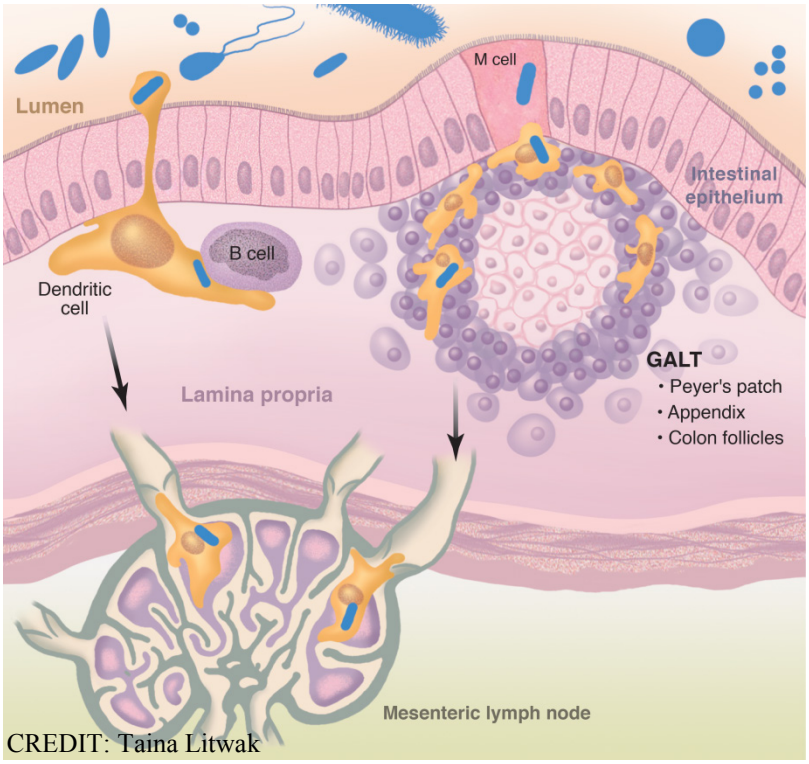

Figure 2. Monitoring of microbiome in the gut lumen by intestinal immune system. (Reproduced from Besselink MG, Dig Sur 2005) Dendritic cells in the LP and PP recognize and take up bacteria from the gut lumen. Subsequently, they present antigens locally in LP and after migration to MLN (23).

5.2 Tolerogenic microbes

Many microbes have different effects on the immune system. Enterogenic pathogens induce strong inflammatory responses within gastrointestinal tract, while commensal bacteria can induce tolerance. During the last years many groups demonstrated that microbiota manipulation can alter cytokine patterns and decrease inflammatory symptoms in colitis and arthritis models. One previously described tolerogenic commensal bacterium is *Bifidobacterium infantis* 35624 (*B. infantis*). We focused on this microbe, which was shown to reduce inflammation in murine models and in human studies, but the molecular mechanism of this effect was unknown.

B. infantis was isolated from resected healthy human gastrointestinal tissue more than decade ago and has been extensively studied for its potential immunoregulatory effects on human cells, such as epithelial cells, DCs and T lymphocytes. For better understanding of its anti-inflammatory properties murine models were used and human trials were conducted.

Epithelial cells create a monolayer, which separates the gut lumen and LP cells. *B. infantis* was shown to adhere to epithelium but it induced neither NF-κB activation nor chemokine secretion (24). Moreover, secretion of CC chemokine ligand (CCL)-20 and interleukin (IL)-8 after stimulation with pathogens or Toll-like receptor (TLR) ligands was reduced in the presence of *B. infantis* (24, 25). These results suggest, that this bacterium can act on epithelial cells, which express limited number of PRRs, inducing anti-inflammatory responses after first contact with gut cells.

The second group of cells, which respond to *B. infantis* are DCs. They are effective in sampling the gut lumen by use of dendrites and after activation some of them migrate from LP to MLN. DCs were isolated from human MLN from Inflammatory Bowel Disease (IBD) patients undergoing surgical resection of the inflamed gut. After stimulation with *B. infantis* they secreted IL-10 and TBF-β, while no IL-12 and tumor necrosis factor (TNF)-α were detected (26). However, these DCs were able to produce both pro-inflammatory cytokines: IL-12 and TNF-α in response to pathogenic bacterium *Salmonella typhimurium*. In contrast, DCs from peripheral blood secreted TNF-α after stimulation with both bacteria, whereas IL-12 was not induced in

both peripheral blood and mucosal DCs. This suggests that the response to commensal bacteria is more specific for intestinal DCs than for non-gut DCs.

As activated DCs can influence further adaptive responses, *B. infantis* effect on tolerogenic T lymphocyte subset was elucidated. Forkhead box P3 (Foxp3) T regulatory (Treg) cells were increased after bacterial administartion in murine models. Moreover, these cells were functional and protected against pathogen or lipopolysaccharide (LPS) induced NF-κB activation (27). Nevertheless, induction of Treg cells seems to be not equally specific and effective for all commensal microbes. Other bacteria, such as *Bifidobacterium breve* AH1205 and *Lactobacillus salivarius* AH102 did not induce Treg cells and did not protect against allergic airway inflammation (28).

B. infantis immunoregulatory properties, including Treg induction were tested in numerous murine models of inflammatory diseases. This bacterium was analyzed in infection models, such as *Salmonella typhimurium* infection. Characteristic strong inflammation, including NF-κB activation, pro-inflammatory cytokines secretion and tissue damage were attenuated by *B. infantis* in Treg dependent manner (27). Moreover, this microbe reduced severity of diverse colitis models, including the IL-$10^{-/-}$, the SCID lymphocyte adoptive transfer and the dextran sodium sulphate (DSS) model (20, 30). Interestingly, *B. infantis* did not loose anti-inflammatory properties in IL-10 deficient mice suggesting that other immunomodulatory molecules secreted by DCs have to play an important role.

Tolerogenic properties of *B. infantis* were also analyzed in humans. Patients with Irritable Bowel Syndrome improved after bacterial consumption (31, 32), confirming results obtained in mice. However, in these studies non-gut related markers were not tested. Recently, the role for specific bacterial strains in orchestrating general tolerance mechanisms was shown. *B. infantis* feeding decreased inflammatory markers in ulcerative colitis and other non-intestine disorders, such as chronic fatigue syndrome and psoriasis (33).

All these results have suggested the strong immunoregulatory properties of *B. infantis*. However, the molecular mechanism leading to an anti-inflammatory cytokine secretion and increase in Tregs was not well understood. A better description

of tolerogenic mechanisms induced by *B. infantis* may result in diverse opportunities in creating commensal bacteria-based therapies for inflammatory disorders treatment and prevention.

5.3 Microbial metabolites in the gut

The gut microbiota is recognized by receptors expressed on gastrointestinal cells and affects the balance between inflammation and tolerance. However, microbes are involved in many other processes taking place in the gut, including production of certain metabolites. The role of the microbiota in digestion, nutrient absorption and production is quite well characterized. Some commensal bacteria in the gastrointestinal tract are an important source of B and K vitamins and vitamins can act on immune system cells (34). Recently, it was discovered that immune cells can recognize vitamin intermediates produced by bacteria. The monomorphic major histocompatibility complex class-I related protein (MR1) is capable to present intermediate products of riboflavin biosynthesis pathway to mucosa-associated invariant T cells (35). Moreover, commensals contribute to regulation of bile acids, which are cholesterol-derived amphipathic molecules solubilizing fat in the small intestine. This process helps to digest, absorb fat and A, D, E, K vitamins. Commensal bacteria convert primary bile acids into secondary acids through dehydration reactions (36) and deconjugate bile acids promoting their elimination.

Some metabolites produced by microbes in the gut can act on immune cells in a direct or indirect manner. However, this emerging area is still not deeply investigated and there is increasing evidence showing bacterial metabolites and nutrients impact on innate and adaptive responses. One of the most investigated bacterial metabolites are Short chain fatty acids (SCFAs), which consist of 1-6 carbons and are produced by commensal microbes in the colon from nondigestible plant-derived polysaccharides. *Bacteroidetes* are major producers of SCFA. Three SCFA are present in human intestinal lumen: butyrate, propionate and acetate (37). Butyrate inhibits histone deacetylases and NF-κB activation (38). Moreover, it serves as a main energy source for colonocytes, influencing the epithelial barrier. Treatment of human peripheral blood cells (PBMCs) with SCFAs decreased monocyte chemotactic protein-1 expression and TNF, interferon (IFN)-γ and IL-10 secretion in response to LPS (39). Butyrate action on DCs diminish their ability to present antigens as major histocompatibility complex II (MHCII), CD80, CD86 and CD40 are downregulated (40, 41). Moreover, DCs treated with butyrate decrease expression of IL-12 and increase IL-10 and IL-23 secretion (40, 41). In co-cultures with T cells both IL-10 and IL-17 production is increased (40).

SCFAs act directly on T lymphocytes causing apoptosis and inhibition of proliferation (42, 43). Cytokine production by non-immune cells is also affected by butyrate. Caco-2 cells produce less IL-8 in response to IL-1β and butyrate than IL-1β alone (44). In humans, IBD patients treatment with butyrate reduced intestinal inflammation (45) and biopsies from lesions responded to butyrate treatment with inhibition of NF-κB activation and lower pro-inflammatory cytokine secretion (46).

GPR43 is a G protein-coupled receptor (GPCR), which recognizes SCFAs, such as propionate and acetate. This receptor is expressed on neutrophils, eosinophils, activated macrophages and innate immune cells. The KxB/N serum-induced arthritis, allergic airway inflammation and murine model of IBD are exacerbated in GPR43-deficient mice ($Ffar2^{-/-}$) (47). Germ-free mice represent a similar phenotype and have very low levels of SCFA (47). Propionate and acetate promote neutrophil chemotaxsis through GPR43 (47). This response is due to an increase in intracellular calcium, decrease in cyclic adenosine monophosphate (cAMP) level and extracellular signal-regulated kinases (ERK)1/2 activation (48). Moreover, $Ffar2^{-/-}$ mice had lower neutrophil infiltration in the colon than wild-type animals in DSS-induced colitis model (49). Another GPCR binding SCFA is GPR41, however its expression is low in neutrophils and monocytes (48, 50), suggesting a limited role in immune system regulation.

5.4 Microbiota alteration in diseases

The role for the microbiota in influencing human health is better understood with every year. It does not only impact the digestive tract but also influences immune responses and metabolism. There is growing evidence suggesting that the gut microbiota contributes to metabolic disorders and pathogenesis of diverse diseases. However, only few examples are discussed in this introduction. It remains unknown whether microbiota alterations are a cause or a consequence of these diseases.

5.4.1 Obesity, metabolic syndrome and diabetes

Metabolic syndrome has become one of the most common diseases in developed countries. It consists of obesity, glucose homeostasis disorders (such as type 2 diabetes), lipid homeostasis disorders and hypertension (50). The gut microbiota has been shown to influence the amount of energy harvested from food and fat storage (51), subsequently leading to changes in host metabolism and energy balance (51-54). In obesity and type 2 diabetes, the microbiota can also contribute to development of low-grade inflammation (55-57). This type of inflammation is common in obesity and type 2 diabetes but is not included in the standard definition of metabolic syndrome.

Firstly, gut microbiota changes were analyzed in genetically obese mice. The outcome was an increase in *Firmicutes* and decrease in *Bacteroides* (52, 58). Moreover, a high-fat diet also reduced *Bifidobacteria*, *Eubacterium rectale*, *Blautia coccoides* and *Bacteroides*-like bacteria levels in murine intestine (55). Many other studies confirmed the *Firmicutes* increase and *Bacteroides* decrease associated with obesity in murine models (53, 59, 60).

In humans, *Firmicutes/Bacteroides* ratio changes connected with obesity are not so clear (61-65), while more specific differences in gender or species were detected. *Bifidobacterium* abundance is lower in obese or type 2 diabetic patients than in healthy, lean individuals (63, 64) and *Faecalibacterium prausnitzii* is decreased in type 2 diabetes (66).

5.4.2 Ulcerative colitis and Crohn's disease

IBD can be divided into two groups: ulcerative colitis, which is associated with T helper (Th)2 cytokines and NK-T cells (67, 68) and Crohn's disease, which is associated with Th1 and Th17 responses. Ulcerative colitis affects the mucosal layer of the colon and inflammation is strongly diffuse, while Crohn's disease is more heterogenous. It is characterized by diverse inflammatory lesions, which can be detected in any part of gastrointestinal tract. Lesions can differ in phenotype e.g. can be inflammatory, penetrating or structuring.

A reduction of species diversity was shown in patients with ulcerative colitis. This effect was associated with instability of dominant taxa, whereas in healthy individuals intraindividual stability and similarity reached approximately 80% (69). In the colon of ulcerative colitis patients increased load of *Desulfovibrio*, bacteria generating sulphides was detected (70). Moreover, *Fusobacterium varium* and *Fusobacterium nucleateum* strains invading epithelium were identified in inflamed colon biopsies (71-73). In contrary, anti-inflammatory species such as *Fusobacterium prausnitzii* was reduced during both active disease (74) and remission.

Similar to ulcerative colitis, it was noted that in Crohn's disease there is an instability of dominant species (75). Specific species changes are more difficult to describe, because they depend on the affected part of the gut. In ileal Crohn's disease *Faecalibacterium* and *Roseburi*a were not present, while *Enterobacteriaceae* and *Ruminococcus gnavus* levels were increased (76). Interestingly, potentially pathogenic adherent-invasive *E. coli* (AIEC) has been isolated form ileal Crohn's patients (77, 78). These bacteria adhere to intestinal epithelial cells, invade them and replicate within macrophages but they do not adhere to enterocytes from healthy individuals (79). Thus, an imbalance between protective and damaging microbes may play a role in influencing mucosal and systemic immunoregulatory responses.

5.5 Microbiota recognition by dendritic cells

DCs are able to recognize a wide variety of microbial structures called pathogen associated molecular patterns (PAMPs), which are characteristic for different groups of microorganisms. They are recognized by PRRs on the surface of DCs, in endosomes or in the cytoplasm. The most well described and phylogenetically conserved group is called TLRs. They recognize components of bacteria, viruses and in some circumstances also self material. The second group of PRRs are NOD-like receptors (NLRs), which are represented by nucleotide-binding oligomerization domain 1 (NOD1) and nucleotide-binding oligomerization domain 2 (NOD2). The third group are RIG-I-like receptors (RLRs). The fourth group of PRRs are C-type lectin receptors (CLRs), which bind carbohydrate structures present on microbiota and host cells. Additional PRR families exist, but will not be discussed further.

DCs are responsible for shaping T cell responses. For this three signals are needed. First is MHC-peptide complex recognition. Second, costimulatory molecules expression and third, secreted cytokines such as IL-10, IL-12, transforming growth factor beta (TGF-β) and other membrane-bound or soluble molecules such as retinoic acid. All of these factors influence the T cell polarization program, which can result in Th1, Th2, Th9, Th17, Th22 or Treg responses. The selection of cytokines and other molecules involved in these processes depends on the manner by which DCs are activated. The final outcome can be the effect of many different PRRs activation on the same cell. There is still not too much known about PRRs cross-talk. For instance, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) signaling modulates TLR signaling through Raf-1 dependent acetylation of p65 via an unknown mechanism (80). DC immunoreceptor (DCIR) after internalization is present in the same compartment as TLR8 and TLR9. In myeloid dendritic cells (mDCs) DCIR signaling influences TLR8-mediated TNF and IL-12 secretion, while in plasmacytoid dendritic cells (pDCs) DCIR decreases TLR9-induced TNF and IFN-α production (81, 82).

5.5.1. Toll-like receptors

There are two classes of DCs in human peripheral blood. Plasmacytoid DCs express TLR7 and TLR9, while myeloid DCs express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR8. TLRs are type I membrane glycoproteins conserved from *Caenorhadbitis elegans* to humans (83). Toll was first identified in *Drosophila* as a protein crucial for antifungal responses (84). TLRs consist of ectodomains containing leucine-rich repeats, which recognize PAMPs; transmembrane domains and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains involved in downstream signaling. TLRs recognize diverse PAMPs, such as proteins, lipids, lipoproteins, nucleic acid from microbes (85). TLR signaling leads to MHC and costimulatory molecule upregulation and cytokine secretion, providing DCs with the ability to prime naïve T cells. TLRs play an essential role in appropriate immunity against infection, however their inappropriate activation leads to acute and/or chronic inflammation. Myeloid differentiation primary response protein 88 (MyD88) deficient mice are more susceptible to DSS-induced colitis, what suggests that commensals can be recognized by TLRs in steady-state condition and contribute to intestinal homeostasis (86). Consistent with this finding antibiotic treatment, which depletes the microbiota, also increased susceptibility to DSS, while oral feeding with LPS and lipoteichoic acid corrected this tendency (86). Interestingly, nonobese diabetic (NOD) mice deficient in innate MyD88 signaling are protected against type 1 diabetes and *Bacteroidetes* species dominate their gut (87). However, this protection is lost, when mice are housed under germ-free conditions. Moreover, some mutations, single nucleotide polymorphism (SNP) in TLR coding genes can cause inappropriate innate immune response and increase susceptibility to many diseases. One of the best known examples are mutations in NOD2, which are recognized risk factors for the development of Crohn's disease.

TLRs can be divided into two groups depending on the cellular localization. The first group is expressed on the cell surface and consists of: TLR1, TLR2, TLR4, TLR5 and TLR6 in humans and recognizes mainly secreted or membrane-bound microbial components. The second group resides in the intracellular vesicles, such as endoplasmatic reticulum (ER), endosomes, lysosomes or endolysosomes. This group includes TLR3, TLR7, TLR8, TLR9 and plays role in microbial nucleic acid sensing. This special and functional separation allows the cell to respond to different PAMPs

from the same microbe. Many pathogens evolved methods to avoid recognition by the innate immune system, for instance intracellular pathogens escape from lysosomes and survive in cytoplasm.

Classically, TLRs are present on antigen presenting cells (APC), such as DCs, macrophages and B cells. In B cell-specific MyD88$^{-/-}$ mice, after DSS treatment, bacteria are found in systemic locations, such as lung. This effect is not present in epithelial cell-specific or DC specific-MyD88$^{-/-}$ mice (88). MyD88 signaling in B cells in steady-state helps to control amounts of IgE in serum and circulating basophiles. When B cells are deficient in MyD88 IgE level is increased in serum and more IgE binds to basophiles (89.) Interestingly, there is growing evidence showing TLRs expression on the surface of T cell subsets (90). This opens new perspectives for direct influence of microbes and/or their metabolites on T lymphocytes. RAG-deficient mice have decreased inflammation after transfer of MyD88-deficient T cells (91). Moreover, CD4+ lymphocytes treated with TLR4 agonist have elevated suppressive function and protect against colitis (92).

5.5.1.1 Surface TLRs

The best described cell surface TLR is TLR4, which responds to LPS from Gram-negative bacteria. In order to bind LPS, TLR4 forms a complex with MD2, however LPS binding requires additional steps. LPS-binding protein (LBP) soluble in the plasma binds LPS. Next LBS is bound by CD14 and finally LPS is delivered to TLR4-MD2 complex (93). In addition TLR4 recognizes other PAMPs, such as *Streptococcus pneumonia* derived pneumolysin, mouse mammary tumor virus envelope protein and respiratory syncytial virus fusion protein (85).

TLR2 is deeply investigated as it recognizes a very wide range of PAMPs. This includes: lipoproteins, peptidoglycan, lipoteichoic acid, lipoarabinomannan from mycobacteria, zymosan, tGPI-mucin from *Trypanosoma cruzi*, hemagluttinin protein from measles virus and polysasccharide A (PSA) (94). Moreover, TLR2 can form heterodimers with TLR1 and TLR6, which increases the number of recognized PAMPs. TLR1 and TLR6 possess different lipid-binding pockets. Therefore TLR1/2 recognizes triacetylated lipoproteins, while TLR2/6 binds diacetylated lipoproteins from Gram-

negative bacteria and mycoplasma. TLR2 is widely described as a PRR mediating host protective immune responses. In general, $TLR2^{-/-}$ DCs are not capable to promote Foxp3 Treg cells development and IL-10 production *in vitro*. *Bacteroides fragilis* produces PSA, which influences maturation of the immune system by promoting development of CD4+ T lymphocytes (95). Foxp3+ Treg cells are activated and produce IL-10. In this way *Bacteroides fragilis* induces protective, immunoregulatory responses in murine model of inflammatory bowel disease and multiple sclerosis (94, 96, 97). All these effects are specifically mediated by PSA acting on TLR2, because PSA does not protect $TLR2^{-/-}$ mice against colitis (98). PSA is delivered to DCs in vesicles, which bud from *Bacteroides fragilis* outer membrane surface (99). Interestingly, PSA enhances the anti-inflammatory activity of Treg cells directly through TLR2 expressed on the surface of CD4+ T lymphocytes (98). Moreover, another commensal microbe *Bifidobacterium breve* is recognized by TLR2 on intestinal CD103+DCs and promotes Tr1 IL-10 producing cells development, however the exact microbial ligand remains unknown (100). TLR2 seems to play important role also in restoring skin homeostasis after injury. Recognition of staphylococcal lipoteichoic acid (LTA) (derived from commensal bacteria) by TLR2 on keratinocytes was shown to attenuate inflammation caused by injury and TLR3 activation. The best described TLR2 ligands, such as Pam3CSK or LTA from different bacteria, were not able to induce the same effect (101). LTA from *Lactobacillus plantarum* L-137 was shown to increase pro-inflammatory IL-12 production and an opposite effect was seen with another strain (102). These differences could be related to LTA D-alanine modifications (101, 102).

TLR5 recognizes flagellin and is highly expressed on CD11b+CD103+ pro-inflammatory DCs in the gut. $TLR5^{-/-}$ mice are non-specifically resistant to *Salmonella*-induced typhoid-like disease, whereas in gastroenteritis gut pathology is exacerbated (103). Interestingly, $TLR5^{-/-}$ mice develop features of metabolic syndrome and transfer of their microbiota to wild-type animals causes similar effects. (104)

5.5.1.2 Intracellular TLRs

TLR3 is an intracellular TLR and is activated by dsRNA, including viral dsRNA produced during replication, genomic RNA of reoviruses and certain small interfering RNA (siRNA) (85). This PRR triggers an antiviral response, mainly type I interferon

secretion. Moreover, TLR3$^{-/-}$ mice are highly susceptible to lethal murine cytomegalovirus infection (105). In humans, TLR3 deficiency increases susceptibility to herpes simplex virus type 1 (HSV-1) (106).

TLR7 and TLR8 recognize ssRNA. Moreover, TLR7 binds synthetic poly(U)RNA and certain siRNA (107). TLR7 can recognize viral RNA and induce type I interferon production. This PRR is expressed by pDCs and requires autophagy for the most efficient PAMP delivery. When autophagy in pDCs is blocked, vesicular stomatitis virus is not sensed correctly (108).

TLR9 recognizes unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) CpG DNA present in bacteria and viruses, such as murine cytomegalovirus, HSV-1 and HSV-2 (109). This TLR plays an important role in pDC and B cell activation. CpG stimulated B cells increase IL-10 production, while pDCs secrete type I interferon and initiate strong antiviral response. Other receptors like CD303 can downregulate this proinflammatory response and increase TLR-9 induced IL-10 secretion.

5.5.1.3 TLR signaling

In TLR signaling TIR-domain-containing adaptor molecules, including MyD88, TIRAP, TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM), are involved. They are recruited by TLRs and activate diverse signaling cascades. MyD88 can be utilized by all TLRs with only one exception – TLR3. In this pathway NF-κB and mitogen-activated protein kinase (MAPK) are activated and proinflammatory cytokines are produced (85). TLR3 and TLR4 can recruit TRIF, which leads to IFN-regulatory factor (IRF)3 activation and type I interferon secretion. TRAM and TIRAP are adaptor molecules, which recruit TRIF to TLR4 and MyD88 to both TLR2 and TLR4, respectively. Interestingly, TLR4 utilizes all 4 adaptors in order to activate both TRIF and MyD88. Moreover, both cascades need to be activated for proinflammatory cytokine production in response to TLR4 ligand binding. In general TLR signaling can be divided into MyD88- and TRIF-dependent pathways (Figure 3).

MyD88 recruits IL-1 receptor-associated kinases (IRAKs): IRAK4, IRAK1, IRAK2 and IRAKM. TNF receptor-associated factor 6 (TRAF6) interacts with activated IRAK and catalyzes polyubiquitination on Lys63 on both TRAF6 and IRAK. This promotes binding of TAB2 and TAB3, which create regulatory complex with the TGF-β-activated kinase 1 (TAK1). Polyubiquitinin chains bind also NEMO, which constitutes a regulatory component of IκB kinase (IKK) complex required for NF-κB activation. Subsequently TAK1 phosphorylates IKKβ and this results in NF-κB activation. This effect is mediated by phosphorylation and degradation of IkB proteins (110). TAK1 activates also MAPKs, ERK1/2, p38 and c-Jun N-terminal kinase (JNK). TRIF also recruits TRAF6 and activates TAK1 leading to NF-κB activation in similar way as in the MyD88-dependent pathway. TRIF binds the adaptor molecule receptor-interacting protein 1 (RIP1), which is polyubiquinated by Pellino-1 after TLR3 ligand binding. Further, TNF receptor type 1-associated DEATH domain protein (TRADD) interacts with RIP1 and these 2 steps are critical for NF-κB activation in response to TLR3 (111, 112).

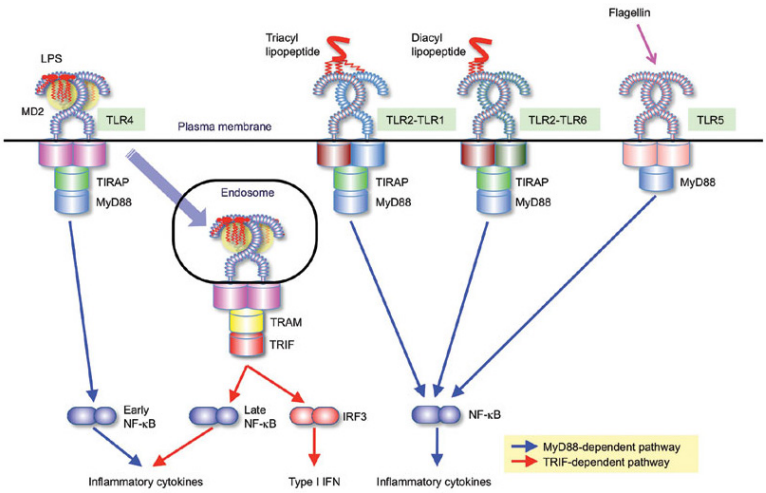

Figure 3. The cell surface TLR signaling. (Reproduced from Kawai T, Nat Immunol 2010) Surface TLRs activate two signaling pathways after ligand binding. MyD88-dependent pathway leads to NF-κB activation and pro-inflammatory cytokines secretion, while TRIF-dependent pathway results in NF-κB activation and pro-inflammatory cytokines production, including type I interferon induction as a result of IRF3 activation. (83)

The TRIF-dependent pathway activates IRF3 and IFN-β production in addition to NF-κB activation (Figure 3, Figure 4) (113). TRIF recruits noncanonical IKKs TBK1 and IKKi necessary for IRF3 activation. In this complex IRF3 is phosphorylated,

dissociate and subsequently trans locates to the nucleus. This complex is functional only in presence of TRAF3, which is important also for TLR7 and TLR9 signaling (114). Moreover, TRAF3 can be involved in inhibition of the MyD88-dependent signaling cascade.

TLR7 and TLR9 signaling require IRF7 and MyD88 activation for type I interferon expression (Figure 4). IRF7 binds MyD88, IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (113). In this complex IRF7 is phosphorylated, dissociates and translocates to nucleus. Moreover, TRAF6 can be involved in ubiquitination of IRF7 resulting in its activation.

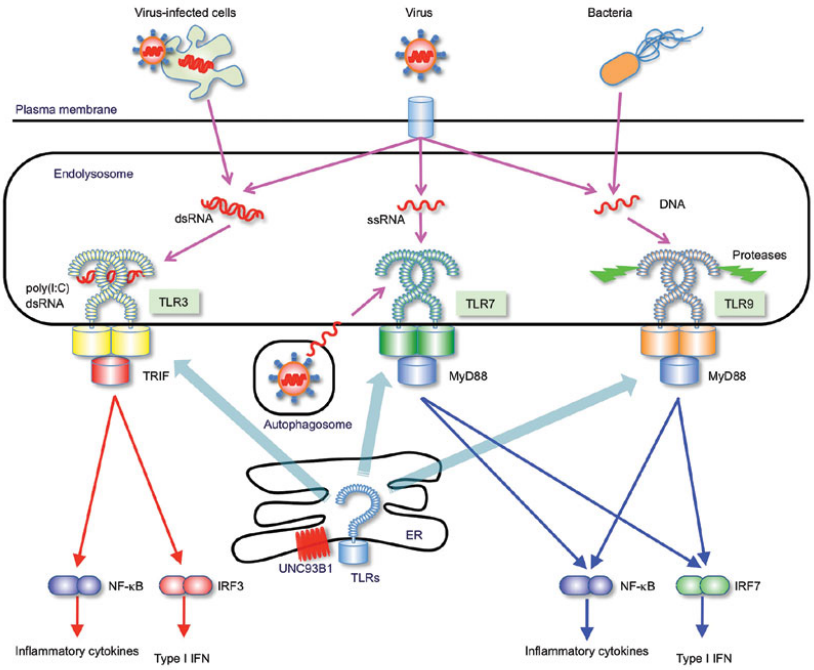

Figure 4. The intracellular TLR signaling. (Reproduced from Kawai T, Nat Immunol 2010) Intracellular TLRs activate two signaling pathways after ligand binding. MyD88-dependent pathway leads to NF-κB and IRF7 activation, inducing pro-inflammatory cytokines secretion. TRIF-dependent pathway results in NF-κB activation and pro-inflammatory cytokines production, including type I interferon induction as a result of IRF3 activation. (83)

Activation of MyD88-dependent pathway influences many genes related to NF-κB transcription. IkBξ is inducible coactivator for NF-κB p50 subunit and is involved in IL-6 and IL-12p40 induction (115). C/EBPδ maximizes IL-6 production (116), while

IkB-NS suppresses both IL-6 and TNF expression. This effect is achieved via modulation of NF-κB p65 subunit binding to DNA (117).

TLR signaling is negatively regulated by a number of inhibitory molecules, such as SOCS1 (suppressor of cytokine signaling 1) or MyD88s (MyD88 short) (118). Moreover, Src homology phosphatase-1 (SHP-1) negatively regulates MyD88-dependent pathway. It suppresses IRAK1 and IRAK2 (119).

5.5.2. NOD-like receptors

The second group of PRRs are NLRs. The NOD subfamily is represented by NOD1 and NOD2, encoded by *card4* and *card15*, respectively. They recognize muropeptide motif mainly from gram-negative bacterial peptidoglycan (PTGN) and muramyl dipeptide (MDP), respectively (120, 121). After ligand binding by NODs NF-κB is activated and pro-inflammatory cytokines are produced. PTGN can enter blood circulation and prime killing of some bacterial pathogens. After bacterial depletion, lower levels of circulating PTGN is detected and neutrophils are less efficient in killing *Streptococcus pneumonia* and *Staphylococcus aureus* (122). NOD2 mutations are known to potentiate TLR-driven NF-κB activation in Crohn's patients. However, NF-κB activation occurs more efficiently through TLRs and NOD2 more specifically induces IL-1β release (123). In contrary, nuclear oligomerization domain proteins subfamily C (NLRC)3 and NLRC5 are less well described and are recently discovered NOD subfamily members. NLRC3 is involved in MyD88-dependent NF-κB activation inhibition via TRAF6 modification (124). NLRC5 expression is induced by IFN-γ, LPS stimulation and viral infections. However, the role of this receptor *in vivo* is not well understood.

The NLRP subfamily consists of NLRP1 to NLRP12. NLRP1, NLRP2, NLRP3 and NLRC4 are well known for their ability to create a complex called an inflammasome (124). Ligand activated receptor interacts with ASC and pro-caspase-1, creating the basic inflammasome unit. The inflammasome unit cleaves pro-caspase-1 to caspase-1, which cleaves pro-IL-1β to active IL-1β. The most investigated is the NLRP3 imflammasome. It is activated by numerous PAMPs, danger-associated molecular patterns (DAMPs) and other factors, such as silica, alum and uric acid

crystals. From various PAMPs β-glucan from *Candida* and *Saccharomyces* was shown to activate NLRP3 inflammasome and led to IL-1β production (125, 126). Moreover, M2 protein from influenza A virus activates this inflammasome (127).

5.5.3. RIG-I-like receptors

The RLR family of PRRs consists of only three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and RIG-I like receptor LGP2. They are responsible for detecting viruses (128). The first two are cytoplasmatic DEx(D/H) box helicases detecting viral products, such as genomic RNA and providing the signal for type I interferon production (128). Moreover, RIG-I can recognize RNA harboring 5'-triphosphate ends (129). In contrast, MDA5 activation is specific to picornavirus infection or long dsRNA, such as poly(I:C) (130, 131).

The signaling cascade for RIG-I and MDA5 includes homotypic caspase activation and caspase recruitment domain (CARD) domain interaction with interferon promoter-stimulating factor 1 (IPS-1) adaptor protein (132). This results in RIG-I and MDA5 recruitment to outer membranes of the mitochondria. The whole signaling complex activates interferon regulatory factors (IRFs) and other transcription factors. Finally, IFN-α and IFN-β secretion is induced (133).

LGP2 does not have a CARD domain and for long time was thought to be negative regulator of RLRs. However, in LGP2$^{-/-}$ mice responses to viruses recognized by RIG-I and to picornaviruses were impaired (134). Finally, it was suggested that LGP2 facilitates virus recognition by other RLRs through its ATPase domain (134). Recently, LGP2 was shown to be required for antigen specific CD8+ T cell responses during West Nile virus infections (135).

5.5.4. C-type lectin receptors

The fourth group of PRRs are CLRs, which bind carbohydrate structures present on microbes and host cells. They are divided into two families: family I (mannose receptor family) – contains several carbohydrate recognition domains (CRDs) in the extracellular domain and family II (asiaglycoprotein receptor family) – contains only one CRD (136). Family II is divided into two subfamilies: DC-associated C- type lectin 1 dectin-1 (CLEC7A) and DCIR (CLEC4A) (Table 1). They play a role not only in antigen capture but also in interactions with other cells.

Family I		
Receptor	**Cells**	**Ligand**
Mannose receptor (CD206)	mDCs and macrophages	High mannose and fucose, sulphated sugars
DEC205 (CD205)	mDCs	Not determined
Family II		
DC-SIGN (CD209)	mDCs	Fucose and mannose
MGL	mDCs and macrophages	Terminal GalNAc
MDL1	Monocytes and macrophages	Not determined
Langerin	Langerhans cells and dermal DC subsets	High mannaose, fucose and terminal GalNAc
Family II, dectin-1 subfamily		
Dectin-1	mDCs, macrophages, monocytes and B cells	β-1,3-glucan
MICL	mDCs, monocytes, macrophages and neutrophils	Not determined
CLEC2	platelets	podoplanin, rhodocytin
DNGR1	BDCA+ DCs, monocytes and B cells	Not determined
CLEC12B	macrophages	Not determined
Family II, DCIR subfamily		
Dectin-2	mDCs, pDCs, monocytes, macrophages, neutrophils and B cells	High mannose
BDCA2 (CD303)	pDCs, monocytes and macrophages	HCV glycoprotein E2, asialo-galactosyl-oligosaccharides with terminal β-1,4- and β-1,3-galactose residues, HIV-1 envelope glycoprotein gp120
Mincle	mDCs, monocytes and macrophages	α-mannose, trehalose dimycolate
DCIR	mDCs, pDCs, monocytes, macrophages, neutrophils and B cells	glycan structures Lewis(b) and Man3

Table1. CLRs classification. Cell specific expression patterns and their ligands are described. (80, 137-141)

CLR family I is represented by mannose receptor and CD205, which is deeply investigated in context of therapeutic vaccines development in the field of autoimmune diseases. Targeting of β-cell antigens to CD205 results in deletion of antigen-specific autoreactive CD8+ lymphocytes in murine model of type I diabetes (142, 143).

The best described CLR representing family II is DC-SIGN/CD209, which binds viruses and bacteria, for instance HIV-1, Ebola and *Mycobacterium tuberculosis* and *Helicobacter pylori* (144). This receptor signaling cascade was shown to interfere with TLR signaling and it is dependent on NF-κB activation. Recent studies on DC-SIGN have shown that mannose or fucose binding can lead to different signaling cascades due to the dynamic regulation of signalosome in the DCs and thereby change the cytokine secretion pattern in a pro- or anti-inflammatory direction (145). DC-SIGN is also involved in secreted immunoglobulin (Ig)A-antigen complex transport in the intestine (146). DC-SIGN has close homologue L-SIGN present on specialized liver and lymph node endothelial cells and both are not expressed on mouse cells. There are several mouse homologs of human DC-SIGN and L-SIGN: mDC-SIGN and mSIGNR1 to mSIGNR4. The most studied is mSIGNR1, which has similar binding characteristics to human DC-SIGN. However, mSIGNR1 is expressed on marginal zone macrophages, peritoneal macrophages and LP DCs (147, 148).

Another CLR family II member, macrophage galactose-type lectin (MGL), recognizes rare terminal GalNAc structures, and interestingly MGL activation interferes with effector T cell cytokine secretion and proliferation via CD45 engagement. In some cases it can lead to T cell death (149).

Dectin-1 is a family II dectin-1 subfamily member and modulate dendritic activation via noncanonical and canonical NF-kappaB activation through Raf-1 and Syk and induce both pro-inflammatory and anti-inflammatory cytokines (145). Dectin-1 recognizes beta-(1,3)-glucans and is the primary fungal PRR. Many fungi are recognized by dectin-1, including *Candida albicans*, *Aspergillus fumigatus*, *Coccidioides*, *Penicillum*, *Saccharomyces* and *Pneumocystis carinii*. Activation of this receptor by curdlan (dectin-1 agonist) or pathogenic fungus leads to Th17 and Th1 differentiation (150). Dectin-1 is necessary for appropriate lung defense during acute *Aspergillus fumigatus* infection. However, during chronic inflammation caused by

constant exposure to this fungus dectin-1 contributes to inflammation via the induction of IL-22 (151). Dectin-1 plays a critical role in the response to *Candida albicans* infection. Activation of this receptor results in IFN-β production in a Syk and IRF5-dependent manner (152). Recently, dectin-1 was shown to be important for commensal fungi recognition. Dectin-1 deficient animals were more susceptible to chemically induced colitis as a result of changes in responses to resident fungi (2). This result is consistent with SNPs in dectin-1, which correlated with very severe cases of ulcerative colitis.

CD303 represents the last CLR group: II family, DCIR subfamily. This receptor is expressed on pDCs and has no murine homologue (153). It inhibits TLR9 induced IFN-α production (154) and TNF-realted apoptosis inducing ligand (TRAIL) (155). CD303 signaling inhibits antigen processing and further T cell responses. It blocks CpG TLR9 stimulation induced CD40 and CD88 expression (156, 157). This subfamily includes dendritic cell immunoactivating receptor (DCAR) and DCAR1, which are not found in humans. They can be activating receptors but their role is not fully understood. DCAR transcripts were detected in bone marrow-derived dendritic cells (BmDCs), lungs, spleen and at low level in the skin and lymph nodes of mice (158). DCAR1 is specific to CD8+ DCs in the spleen and thymus and some CD11b+ cells in the spleen and bone marrow (159).

5.5.5. Plasmacytoid and myeloid dendritic cells

As mentioned previously, pDCs and mDCs express different sets of PRRs. This enables them to recognize diverse PAMPs from the same microbe and coordinate adaptive response in a proper way. These two subsets of DCs can be detected by use of specific markers. Moreover, pDCs have a morphology similar to plasma cells and only after activation they change shape and dendrites are visible.

Human blood contains two mDC subsets: CD141+ and CD1c+. Classicaly CD1c+ mDCs are $CD11c^{hi}$, CD11b+, while CD141+ mDCs are $CD11c^{low}$ and CD11b-. Human pDCs express on their surface CD123, CD303 and CD304 (Figure 5). Moreover, CD141+ mDCs are involved in cross-presentation and interact with CD8+

T lymphocytes. In contrast CD1c+ mDCs present antigens on MHC II to CD4+ T cells. pDCs are known to be very efficient source of type I interferons.

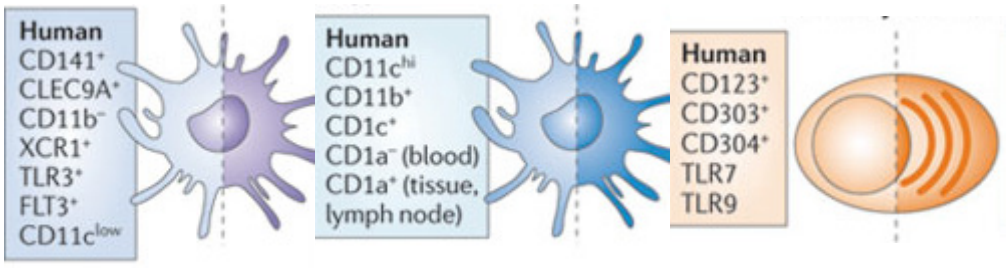

Figure 5. Human blood dendritic cell populations and their markers. (Reproduced from Collin M, Nat Rev Immunol 2011) Myeloid dendritic cells are divided into two subsets: CD141+ and CD1c+. Plasmacytoid dendritic cells express CD123, CD303 and CD304. (160)

5.6 Dendritic cell metabolic immunoregulatory factors

Activated DCs secrete anti- or pro-inflammatory cytokines and upregulate CD80, CD86 and MHC II expression. However, there are many immunoregulatory factors, which can be produced by DCs in response to PAMPs and other endogenous or exogenous stimuli, such as adenosine and histamine. For instance cAMP can be elevated after certain GPCRs activation and inhibit antigen presentation and IL-10 secretion. The best described DC metabolic immunomodulatory factors are indoleamine-2,3-dioxyganese (IDO) and retinoic acid, which are discussed in detail in next sections.

5.6.1. IDO

IDO is an enzyme involved in tryptophan catabolism. It is the first enzyme in kynurenine pathway and it transforms L-tryptophan into N-formylkynurenine (161). IDO is encoded by two genes Ido1 and Ido2, both expressed in mice and humans (162, 163). In the promoter regions response elements for interferon type I (IFN-α, IFN-β) and interferon type II (IFN-γ) are present (164). Moreover, IDO has nonenzymatic properties and acts as a signaling protein in long-term TGF-β driven tolerance (165).

IDO activity and physiological importance was described by many groups. This enzyme plays a protective role in fetal allografts during murine pregnancy, which was confirmed by use of IDO inhibitor 1MT (166). Moreover, blocking IDO activity led to exacerbation of autoimmune, allergic and infectious diseases in murine models (167, 168). In fact, glycosylated allergens can downregulate the level of IDO via the mannose receptor, suggesting that CLRs can play an important role in Treg differentiation (169). This makes IDO an attractive target for immunotherapy (168). Recently it was shown that CpG-induced IDO activity in pDCs was necessary for Treg suppressor activity and blocked their switch to Th17-like cells in the presence of pro-inflammatory mediators (170). Moreover, IDO expressed by pDCs blocked the response to melanoma antigens in lymph node (LN) (171). Tumor clearance was improved by IDO inhibition, which led to IL-6 upregulation and T regulatory cells conversion into Th17 cells (172). Type I autoimmune diabetes in NOD female mice was exacerbated after pDC depletion and

after IDO inhibition (173). Furthermore, pre-diabetic NOD female mice have a defect in IDO expression, which may predispose them to disease development (174).

It is worth mentioning that IDO can bridge multiple psychological responses, such as stress and immune suppression. LPS or chronic inflammation induced IDO causes neurologic depression symptoms and anxiety-like behavior (175, 176). In contrast, acute stress induces low-grade inflammation, which turns on transient tryptophan catabolism dependent on IDO, especially Ido1 gene expression (177). Stressful episodes, prolonged lack of tryptophan and kynurenines formation are immunosuppressive. Furthermore, when tryptophan is utilized by IDO, serotonin production is affected, which can influence subsequent mood alterations and depression development (178).

5.6.2. Retinoic acid

Retinoic acid is present in two different isomeric forms in mice and humans: All-trans-retinoic acid is the most abundant form, whereas 9-*cis*-retinoic acid is present at significantly lower concentration. Retinoic acid takes part in many diverse biological processes such as cellular differentiation, apoptosis, embryonic development, spermatogenesis, and vision (179, 180).

5.6.2.1 Retinoic acid metabolism

Vitamin A (retinol) is obtained from the diet in three forms: all-trans-retinol, retinyl esters or β-carotene and transported in the blood as a complex with retinol-binding protein (RBP) and transthyretin (TTR) (181, 182). Small intestine epithelial cells convert vitamin A to retinyl esters, which are transported in chylomicrons via the lymph into the bloodstream. In the liver retinyl esters are taken up and stored in the stellate cells (181, 182). Retinoic acid is produced within the cell from vitamin A in two tightly regulated steps (Figure 6) (183, 184). Retinol and β-carotene are oxidized to retinaldehyde (retinal) and further retinal is converted to retinoic acid. In first steps many ubiquitously expressed aldehyde dehydrogenases (ADHs) and short chain dehydrogenase reductases (SDRs) are involved. In contrast, retinaldehyde dehydrogenases family (RALDH), which irreversibly oxidizes retinal to retinoic acid, expression is limited to certain cell types, such as intestinal epithelial cells (185, 186),

nerve fibers (187), MLN stromal cells and DCs in PP, MLN, LP (185, 188, 189). Splenic and peripheral lymph node DCs express these enzymes at a very low level (189). 3 enzymes belonging to RALDH family were well described: RALDH1, RALDH2 and RALDH3. RALDH4 is present in mice but its physiological role is unknown (190). RALDH2 and RALDH3 knockouts are lethal (191-193). Only RALDH1 knockout is not so critical for survival (194). This enzyme is mainly produced by epithelial cells and has been recently associated with possible protection from asthma development. RALDH2 is present in MLN and LP $CD103^+$ DC subsets (195), while RALDH3 can be detected in PP DCs. In addition to retinoic acid production these enzymes directly regulate lipid metabolism. Retinoic acid is released from cells and acts in paracrine or autocrine fashion. Moreover, retinoic acid is catabolized in the cytoplasm by the CYP26 class of P450 enzymes and its metabolites, such as 4-hydroxy retinoic acid and 4-oxo retinoic acid, are eliminated with bile and urine. Retinoic acid induces its own degradation by CYP26A1 upregulation (196).

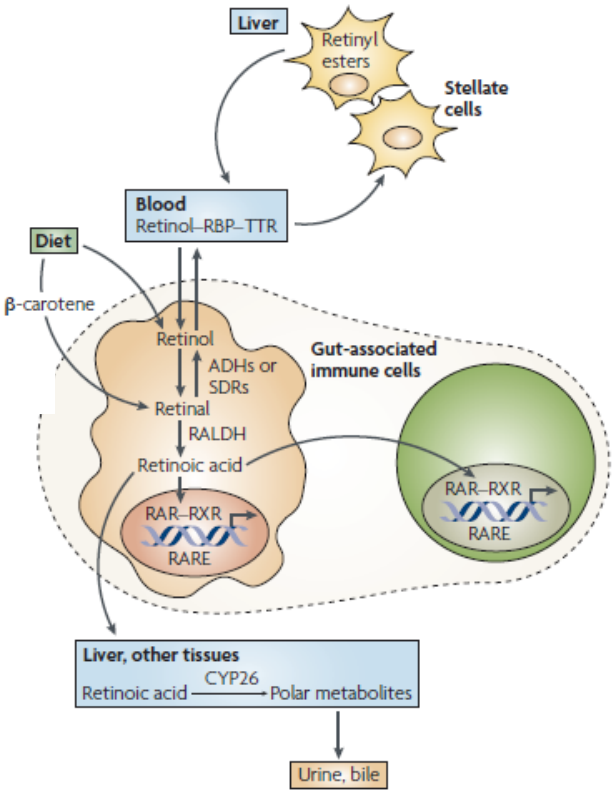

Figure 6. Vitamin A (retinol) metabolism in the small intestine. (Reproduced from Mora JM, Nat Rev Immunol 2008) Retinol is transported in the blood as a complex with retinol-binding protein (RBP) and transthyretin (TTR). It can be stored in the liver as a retinyl ester. In the small intestine retinol is converted into retinal by aldehyde dehydrogenases (ADHs) or short chain dehydrogenase reductases (SDRs). Next step is irreversible and results in retinoic acid production by retinaldehyde dehydrogenases enzymes (RALDH). Retinoic acid interacts with retinoic acid receptor (RAR) and the retinoid X receptor (RXR) and subsequently this complex binds to specific retinoic acid response element (RARE) in the promoter regions of retinoic acid-dependent genes. Retinoic acid is catabolized in the liver and other tissues by the CYP26 and its metabolites are eliminated with bile and urine. (197)

5.6.2.2 Retinoic acid receptors

Retinoic acid acts on different cells by binding to retinoid nuclear receptors, such as retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (198). Each class of retinoid receptor has three main isoforms, namely α, β, and γ, encoded by separate genes. Furthermore, each gene has multiple splice variants (198). These receptors form heterodimers (RAR/RXR) or homodimers (RXR/RXR). They are known to regulate more than 500 genes (199). RARs are ubiquitously expressed and upregulated by retinoic acid (181, 182). RXRs form homodimers, which are specific receptors for *9-cis*-retinoic acid. This form of retinoic acid can also signal through

RAR/RXR heterodimers. In contrast, all-trans-retinoic acid binds only to RARs (198). RXRs can interact with other nuclear receptors, such as thyroid hormone receptor, peroxisome proliferator activated receptor (PPAR) and liver X receptor (200). RAR/RXR complexes bind to specific retinoic acid response element (RARE) in the promoter regions of retinoic acid-dependent genes (Figure 6). They function as transcriptional enhancers or repressors (198, 201). Moreover, retinoic acid can have a direct influence on the activation of many transcription factors through the phosphorylation of the cAMP response element-binding protein (CREB), the mitogen activated kinase ERK1/2, JNK and p38 in neuronal cells (202-204). Retinoic acid inhibits the induction of c-Jun and c-fos (205). Interestingly, retinoic acid interacts and signals through the PPARβ (also known as PPARδ) nuclear receptor (197, 206). The ratio of cellular retinoic acid-binding proteins (CRABPs) to fatty acid-binding protein 5 (FABP5) determines if signaling occurs through RAR or through PPARβ (206). A high CRABP:FABP5 ratio promotes RAR signaling, which results in growth inhibition and apoptosis in some cell lines. In contrast, a low ratio favors FABP5-mediated delivery of retinoic acid to PPARβ and survival in the same cells (206).

5.6.2.3 Retinoic acid production by intestinal CD103+ DCs

DCs in the intestinal mucosa play an important role in regulating the balance between tolerance and immunity. They recognize antigen/microbiota, migrate to MLN and induce appropriate responses by priming naïve T cells. DCs are present in the GALT and LP of the small intestine, while in the large intestine they are mainly placed in lymphoid aggregates. CD103+ DCs are a major DC subset in the small intestinal LP. In steady state, they continuously migrate to MLN, where they promote tolerance. Murine small intestine LP and MLN CD103+ DCs are capable of converting retinol into retinoic acid. They express a higher level of Aldh1a2 (gene encoding RALDH2) and possess higher RALDH activity than DCs from other tissues, including CD103+ DCs from the colon (189, 207- 210). CD103+ DCs from colon express similar levels of RAR α, β and γ as CD103+ DCs from small intestine (211). However, lower retinoic acid production in the colon can be related to retinol (substrate) concentrations, which are significantly higher in the small intestine and MLNs than in the colon, spleen, serum and peripheral lymph nodes (PLNs) (210, 211).

MLN CD103+ DCs ability to promote retinoic acid metabolism is closely related to their tolerogenic properties (Figure 7). They induce gut homing receptors, such as CC chemokine receptor 9 (CCR9) and α4β7 integrin expression on T and B cells (185, 211-213), enhance TGF-β-dependent Foxp3+ T regulatory cells priming, while suppressing Th17 differentiation (189, 213, 214). Moreover, it has been shown that they promote the generation of IgA secreting cells (215). It is worth noting that RALDH+ DCs are present in other tissues e.g. skin (207). Cutaneous RALDH+ DCs are CD103- and do not induce gut homing receptor expression.

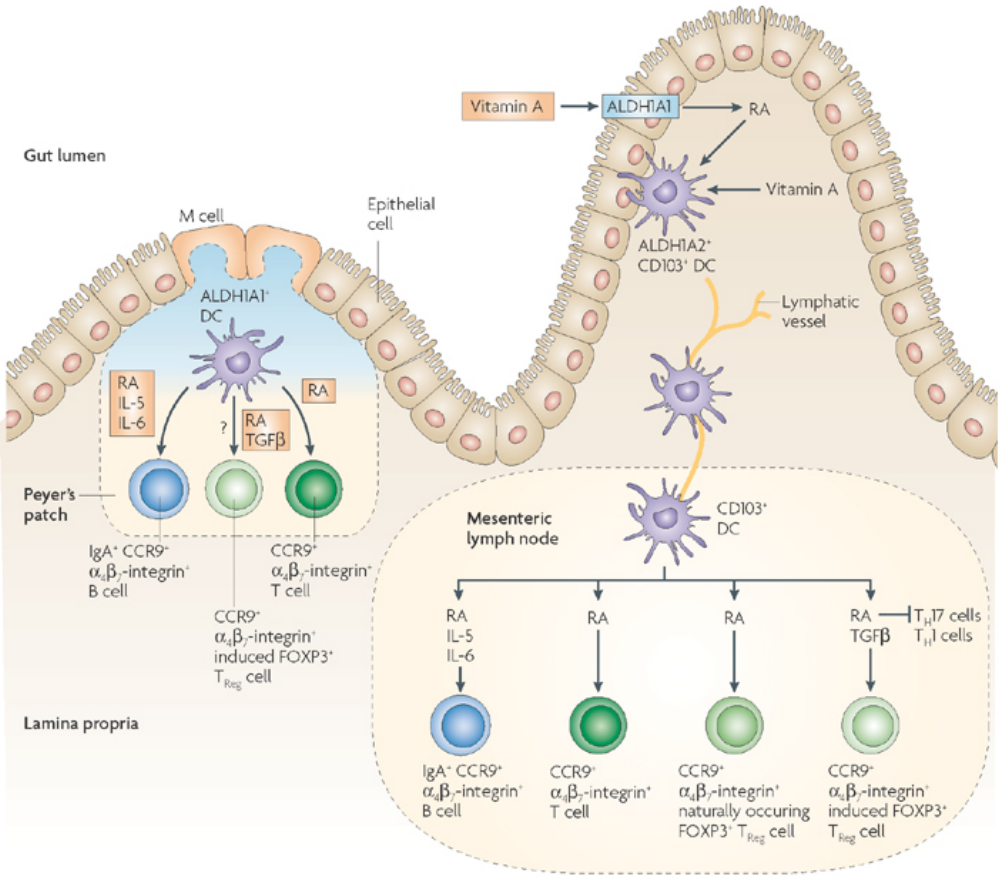

Figure 7. Role of retinoic acid in the small intestine. (Reproduced from Coombes JL, Nat Rev Immunol 2008) In the murine small intestine many cells are capable of converting retinol into retinoic acid (RA). Epithelial cells and PP dendritic cells (DCs) express Aldh1A1, while MLN CD103+ DCs express Aldh1A2. Intestinal CD103+ DCs ability to promote retinoic acid metabolism is tightly related to their tolerogenic properties. They induce gut homing receptors, such as CCR9 and α4β7 integrin expression on T and B cells, enhance TGF-β-dependent Foxp3+ T regulatory cells priming, while suppressing Th17 and Th1 differentiation. Moreover, they promote the generation of IgA secreting cells. (216)

As vitamin A is a substrate for retinoic acid production, dietary vitamin A impact on intestinal CD103+ DCs was assessed in vitamin A deficient mice (VAD). These mice are kept on vitamin A deficient diet from embryonic day 11 until adulthood. This deficiency dramatically reduced Aldh1a2 expression and RALDH activity in CD11c+ MLN DCs (217), which were mainly CD103+ (195, 211). At the same time, CD103+ DCs frequency was not changed in the whole gut (195). Both vitamin A and retinoic acid oral supplementation restored this activity (195, 210, 218).

As mentioned earlier, cells other than DCs are involved in retinoic acid metabolism in the gut. The small intestine epithelial cells can convert retinol (219) and have been correlated with tolerogenic DCs imprinting (*in vitro* experiments) (220, 221). Moreover, murine macrophages express Aldh1a2 gene and possess RALDH activity but at lower level than CD103+ DCs (207, 222).

5.6.2.4 Regulation of retinoic acid metabolism

Retinoic acid metabolism has been shown to be regulated through TLR signaling. Many PAMPs including TLR2 homodimer ligands, such as zymosan and Pam-2-cys, TLR2/TLR1 heterodimer ligand Pam3CSK4 and TLR2/TLR6 heterodimer ligand follistatin induced RALDH activity and Aldh1a2 expression (207, 210, 223, 224). The signaling cascade leading to vitamin A metabolism enhancement is not clear, because various TLR2 homodimer and heterodimer ligands activated different pathways. Pam3CSK4 mediated JNK/MAPK signaling and pan-JNK inhibitor decreased vitamin A metabolism (223), whereas zymosan acted via ERK (Figure 8) (224). Nevertheless, TLR signaling alone seems to be not sufficient for Aldh1a2 induction in the small intestine DCs and many studies have showed contradictory results. MLN CD103+ DCs in one study presented normal RALDH activity in $MyD88^{-/-}$ and $TRIF^{-/-}$ mice (195), while others have found small but a significant decrease in RALDH activity in $MyD88^{-/-}$ mice (223) and $MyD88^{-/-}TRIF^{-/-}$ mice (207), which could be related to lower RARβ expression and impaired autocrine retinoic acid effect on DCs. Moreover, TLR-dependent regulation seems to work only through TLR2 as TLR4 and TLR6 did not affect vitamin A metabolism in MLN CD103+ DCs. Only DCs from $TLR2^{-/-}$ mice had reduced metabolism and ability to induce gut homing receptors on T cells (223). Albeit the role for TLR2 remains controversial, because of low level expression on the surface of the small intestine CD103+ DCs (225, 226).

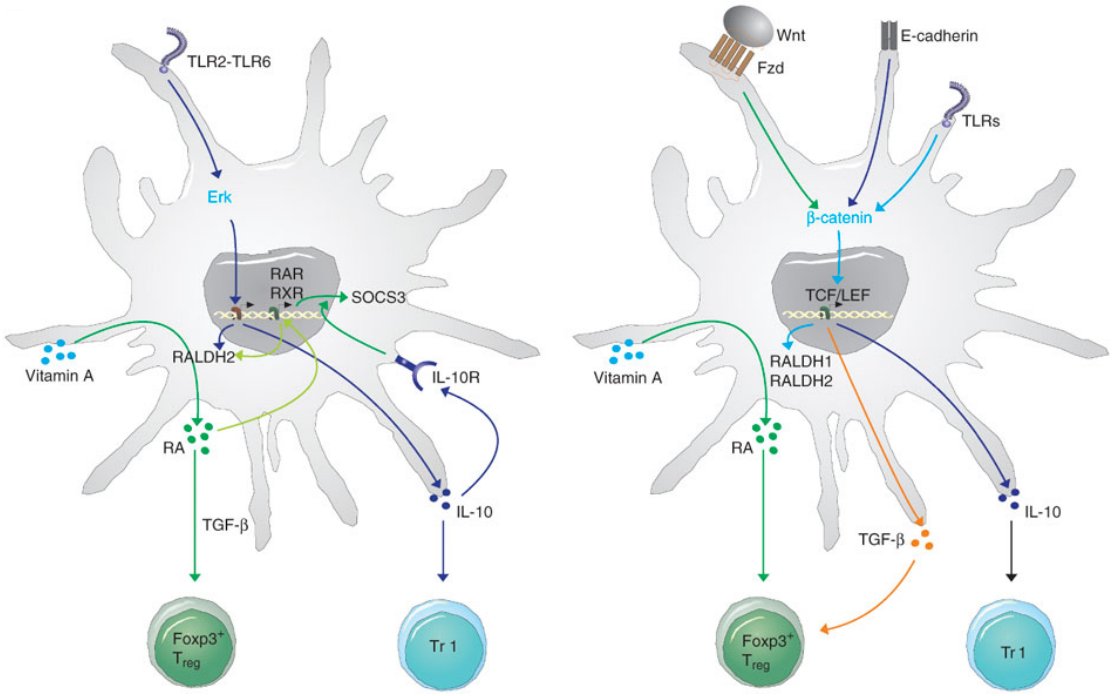

Figure 8. Molecular pathways involved in vitamin A metabolism induction. (Reproduced from Pulendran B, Nat Immunol 2010) Ligand binding to TLR2/TLR6 leads to ERK activation, which results in retinaldehyde dehydrogenase 2 (RALDH2) induction and retinoic acid (RA) production. RA exerts autocrine effect on DC by binding to retinoic acid receptor (RAR) and the retinoid X receptor (RXR). This leads to RALDH2 and suppressor of cytokine signaling 3 (SOCS3) upregulation. IL-10 also enhances expression of both RALDH2 and SOCS3. Moreover, activation of β-catenin pathway by TLRs, Wnt ligands or E-cadherin promotes anti-inflammatory factors, such as RA, IL-10 and TGF-β production. Further, IL-10, RA and TGF-β contribute to Treg and Tr1 cells induction. (227)

Other potential regulators of vitamin A metabolism in CD103+ DCs were tested in *in vitro* assays and murine models. Cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4 and IL-13 induced RALDH activity in BmDCs, splenic DCs and human MDDCs (217, 228). This finding was confirmed *in vivo* in MLN CD11c+ DCs from mice deficient in the GM-CSF/IL-3/IL-5 subunit receptor (217). Albeit these mice had reduced numbers of intestinal CD103+ DCs (229) and another study has shown no alteration in retinoic acid metabolism in MLN DCs (223). The role of IL-4 has not been clearly described. One study suggested lower Aldh1a2 expression in IL4ra$^{-/-}$ mice (230), while another one suggested no changes in RALDH activity (217). Furthermore, PPARγ agonists, such as rosiglitazone, induce vitamin A metabolism in human MDDCs (206) and murine splenic DCs (231) through upregulation of retinol dehydrogenase 10 (RDH10), RALDH2 and CRABP2 expression (232).

Retinoic acid can directly induce RALDH activity and RALDH2 expression in BmDCs and splenic DCs in an autocrine manner. These cells can further promote gut homing receptor expression on T cells and Treg differentiation (195, 211). The signaling cascade, which potentiates retinoic acid metabolism is still not well understood. Retinoic acid self-upregulation requires protein synthesis and is blocked by cycloheximide (translational inhibitor) (210). ERK seems to be involved, because its inhibitor UO126 reduces RALDH activity in MLN DCs (210). Many other factors were tested in order to elucidate signaling cascades leading to retinoic acid metabolism or inhibition. The role of prostaglandin E2 (PGE2) on RALDH2 expression in DCs was investigated. PGE2 inhibited RALDH2 expression in BmDCs stimulated with cytokines. Furthermore, lower levels of PGE2 resulted in changes in RALDH activity in splenic DCs (228). The proposed suppression mechanism includes inducible cAMP early repressor (ICER) expression, which was detected in MLN and peripheral lymph node (PLN) DCs displaying RALDH activity. However, PGE2 levels are similar in non-gut tissues and thus PGE2 does not seem to be critical regulator of vitamin A metabolism. Recently, β-catenin deficiency in CD11c+ cells was shown to induce an inflammatory pattern in LP DCs and macrophages (233). Small intestine CD103+ DCs had reduced RALDH2 expression suggesting interaction between WNT-β-catenin signaling and pathways regulating retinoic acid metabolism (Figure 8). However, cellular regulation of vitamin A metabolism remains unknown.

5.6.2.5 Retinoic acid direct effect on DCs

Retinoic acid has direct effect on DCs. It regulates antigen presentation, increases matrix metalloproteinases, which leads to more efficient migration to lymph nodes and was described to potentially boost specific T cell responses against tumors (234). In inflammatory condition (for instance high levels of TNF-α) retinoic acid signaling through RXRs enhances DC maturation and antigen presentation capacity (235). Moreover, if DCs are pre-treated with retinoic acid, they are able to store this metabolite (236) and release it in close proximity to other cells, such as T cells.

5.6.2.6 Retinoic acid effects on T and B lymphocytes

Retinoic acid affects many T cell properties, such as cytotoxicity (237) and proliferation (238). Furthermore, studies in VAD mice have revealed defects in T helper cells activity (239), which can be explained by impairment in competing with 1,25-dihydroxyvitamin D3 for RXR. More specific regulation by retinoic acid seems to influence the balance between Th1, Th2 subsets and especially Treg cells and Th17 subset.

Vitamin A deficiency in mice has been correlated with decreased Th2 responses (240), while vitamin A supplementation blocked Th1 effector cytokines (241, 242). Interestingly, retinoic acid can promote Th2 differentiation by inducing IL-4 and GATA, MAF and signal transducer and activator of transcription 6 (STAT6) expression and decreasing T-bet (241, 243, 244). In the mouse model of asthma vitamin A supplementation increased disease severity (245).

Regulation of Th17 and Treg cell balance by retinoic acid has been intensively investigated. TGF-β supports Treg generation in the periphery (246), albeit retinoic acid can enhance it significantly (Figure 9) (215, 247) by acting on RARα (248). Furthermore, retinoic acid inhibits Th1 and Th17 development (215, 249, 250). Especially effective in this process are DCs from GALT and the small intestine (189, 214 Moreover, retinoic acid upregulates gut homing receptors CCR9 and α4β7 expression on activated T cells (Figure 9) (214, 247). These cells migrate to the gut in response to CCL-25 and mucosal addressin cellular adhesion molecule (MAdCAM). The role of retinoic acid was confirmed in several *in vitro* assays. 1nM retinoic acid induced α4β7 integrin on T cells. In contrast, RALDH inhibition by citral or RAR signaling inhibition by LE540 blocked CCR9 and α4β7 expression on T lymphocytes (185). Furthermore, LP, PP, MLN CD103+ DCs imprinted naïve T cells and B cells (251) with the ability to migrate to the gut (185, 213, 230). It is worth mentioning that this migration is specific for small intestinal and not for colonic DCs (252).

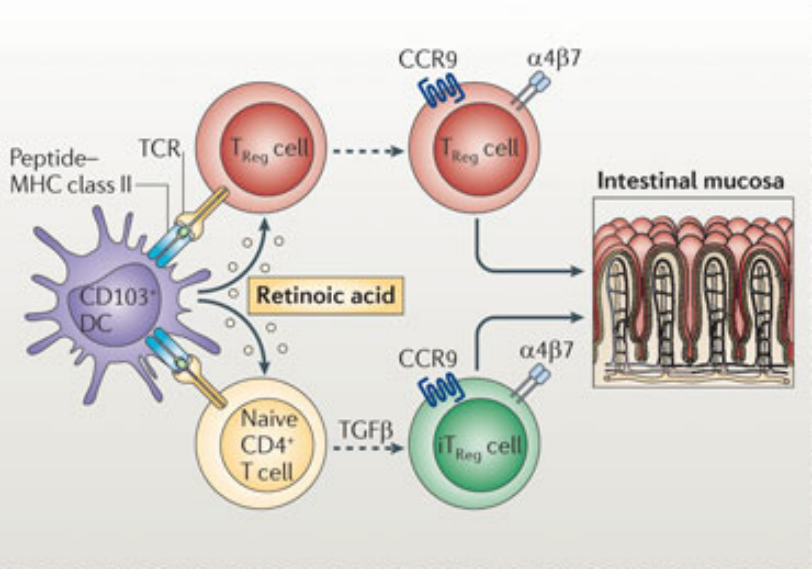

Figure 9. Retinoic acid induces gut homing receptor expression on Treg cells. (Reproduced from Campbell DJ, Nat Rev Immunol 2011) Retinoic acid produced by intestinal CD103+ dendrtitic cells (DCs) induces CCR9 and $\alpha_4\beta_7$ expression on naturally occurring thymus-derived Treg cells and on *de novo* differentiated inducible Treg (iTreg) cells. Moreover, retinoic acid supports TGF-β during iTreg induction. (253)

Vitamin A metabolism in the gut also regulates B cell functions, such as class switching to IgA (213) antibody production in response to bacterial antigens. Depending on local conditions, retinoic acid can act on other B cell features, for instance inhibit B cell proliferation, enhance B cell activation (236, 254) or inhibit B cell apoptosis. All these effects are mediated through RARs (255). Retinoic acid influence on class switch was analyzed by many groups. The presence of retinoic acid induces IgA secretion by LPS activated splenocytes (256) or B cells stimulated with LPS co-cultured with splenic DCs (257). However, retinoic acid, without DCs, during purified B cells activation is not sufficient for IgA class switch (213, 257). Also required are IL-5 (213, 256) or IL-6 (213, 258), which are generally involved in IgA production (231, 259). Moreover, inducible nitric oxide synthase (iNOS), whose promoter contains RARE (260), and nitric oxide also contribute to IgA class switch (261). Albeit, experiments done in VAD mice have shown that retinoic acid is not needed for IgA+ antigen specific cells (ASCs) generation in other mucosal compartments than gut. Numbers of IgA+ ASCs were decreased in the small intestine (213, 262), while serum levels stayed normal (213).

5.7 Immunoregulation by microbes

As it was discussed in this introduction, microbiota in the gastrointestinal tract impacts many physiological processes. It has been shown to influence digestion, nutrient absorption, host metabolism and immune function. In many diseases, microbial composition in the gut is changed, however it remains unknown if this alteration causes disease or it is a consequence of a prolonged inflammatory condition. Tolerogenic microbes help to keep the balance between anti- and pro-inflammatory responses and may be beneficial in the treatment of the gut and non-gut inflammatory disorders. The complicated multicellular signaling networks activated by commensal microbes are involved in this process and require further detailed investigation. This thesis is focused on developing a better understanding of a specific commensal bacterium *Bifidobacterium infantis* 35624, which has been previously shown to possess strong immunoregulatory properties.

6. Aims

B. infantis is commensal bacterium which has been previously shown to protect against inflammatory diseases in murine models e.g. colitis and respiratory allergy. The protective host immune response in murine models was demonstrated to include the induction of T regulatory cells. However, the molecular basis for the Foxp3 regulatory T cells induction by specific microbes is poorly understood and was not deeply investigated for this microbe.

The aims of this thesis were: (1) to elucidate the molecular mechanisms responsible for *B. infantis* recognition and monocyte derived dendritic cells (MDDCs), myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) activation; (2) To analyze the effect of activated dendritic cell subsets on naive T cell priming; (3) to determine if *B. infantis* induces retinoic acid production by mucosal $CD103^+$ DCs; (4) to analyze the role of *B. infantis*- induced retinoic acid metabolism in protection against DSS-induced colitis.

7. Results

7.1 *Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood; potential role for myeloid and plasmacytoid dendritic cells

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7.2 Immunomodulation by *Bifidobacterium infantis* 35624 in the murine lamina propria requires retinoic acid-dependent and independent mechanisms

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ABSTRACT

Appropriate dendritic cell processing of the microbiota promotes intestinal homeostasis and protects against aberrant inflammatory responses. Mucosal $CD103^+$ dendritic cells are able to produce retinoic acid from retinal, however their role *in vivo* and how they are influenced by specific microbial species has been poorly described. *Bifidobacterium infantis* 35624 (*B. infantis)* feeding to mice resulted in increased numbers of $CD103^+$retinaldehyde dehydrogenase $(RALDH)^+$ dendritic cells within the lamina propria (LP). $Foxp3^+$ lymphocytes were also increased in the LP, while T_H1 and T_H17 subsets were decreased. 3,7-dimethyl-2,6-octadienal (citral) treatment of mice blocked the increase in $CD103^+RALDH^+$ dendritic cells and the decrease in T_H1 and T_H17 lymphocytes, but not the increase in $Foxp3^+$ lymphocytes. *B. infantis* reduced the severity of DSS-induced colitis, associated with decreased T_H1 and T_H17 cells within the LP. Citral treatment confirmed that these effects were RALDH mediated. $RALDH^+$ dendritic cells decreased within the LP of control inflamed animals, while $RALDH^+$ dendritic cells numbers were maintained in the LP of *B. infantis*-fed mice. Thus, $CD103^+RALDH^+$ LP dendritic cells are important cellular targets for microbiota-associated effects on mucosal immunoregulation.

INTRODUCTION

The mammalian gastrointestinal microbiota is required for optimal host development and ongoing immune homeostasis [1-3]. The microbiota aids in the digestion of foods, competes with pathogens, degrades mucin and promotes the differentiation of epithelial cells and mucosa-associated lymphoid tissue. In addition, the composition and metabolic activity of the microbiota has profound effects on proinflammatory activity and the induction of immune tolerance by influencing a broad range of mucosal cell types including epithelial cells, dendritic cells, iNKT cells and T lymphocyte subset activity [4-6].

Gastrointestinal immune homeostasis is dependent on a number of local conditioning factors that reduce pathological proinflammatory responses to non-pathogenic microbes. For example, epithelial-derived cytokines such as TSLP and IL-25 limit dendritic-cell secretion of IL-12 and IL-23, while promoting IL-10 secretion [7]. In addition, certain dendritic cell subsets within the mucosa can metabolize vitamin A into retinoic acid, such as the $CD103^+$ dendritic cell subset [8, 9]. Retinoic acid is synthesized from stored or dietary retinol by the oxidation of retinol to retinal, followed by oxidation of retinal to retinoic acid. The final step is catalyzed by aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1) and ALDH1 subfamily A2 (Aldh1a2), also called RALDH enzymes. 3,7-dimethyl-2,6-octadienal (citral) blocks RALDH enzymatic activity. Dendritic cell-derived retinoic acid has dramatic effects on dendritic cell activity and lymphocyte subset plasticity. Retinoic acid can have seemingly conflicting effects on lymphocyte polarization, such as promoting T_H17 cells or T_{reg} cells [10]. The promotion of T_H17 versus T_{reg} phenotypes may be related to the local concentration of retinoic acid, the dendritic cell subset secreting retinoic acid, the local level of pro-inflammatory mediators and TGF-β, concomitant toll-like receptor activation or induction of specific microRNA [11-14]. So far, the role of specific microbial species in influencing retinoic acid metabolism and $CD103^+RALDH^+$ dendritic cells *in vivo* has been poorly understood.

Bifidobacterium longum subsp. *infantis* 35624 (*B. infantis*) was originally isolated from resected human healthy gastrointestinal tissue and human clinical studies have demonstrated its efficacy in Irritable Bowel Syndrome patients [15, 16]. In addition, murine studies have demonstrated that this microbe protects against inflammatory disorders across a range of inflammatory conditions including colitis, pathogen infection, arthritis and respiratory inflammation [17-20]. Previously, *in vitro* studies with

human dendritic cells suggested that promotion of retinoic acid metabolism by *B. infantis* was a key regulatory feature of this bacterium [21]. In this report, we demonstrate that *B. infantis* feeding to mice results in increased $CD103^{+}RALDH^{+}$ dendritic cells within the mucosa, which are responsible for the suppression of T_H1 and T_H17 lymphocytes and amelioration of dextran sulfate sodium (DSS)-induced colitis.

METHODS

Bacteria and animal models

Wild-type C57BL/6 mice were obtained from Charles River and maintained under specific pathogen free conditions. Mice were housed at the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the duration of the study, and all experimental procedures were carried out in accordance with Swiss law. Experimental protocols were approved by the Ethics Committee of the "Amt für Lebensmittelsicherheit und Tiergesundheit Graubünden", application number 2011-15. In the first experiment, three groups of mice were utilized (n=8 per group). Group 1 did not receive any bacterial supplementation, while groups 2 and 3 were fed *B. infantis* for 7 days. Each day lyophilized bacteria were resuspended in sterile water to final concentration of $6x10^8$ colony forming units (cfu)/ml. For group 3, 2mg of citral (Sigma, St. Louis, USA) was dissolved in 10% DMSO (Sigma) and was injected i.p. daily in order to suppress retinoic acid metabolism.

In the dextran sodium sulfate (DSS) colitis model, five groups of wild-type C57BL/6 mice (n=8 per group) were utilized. Group 1 was the negative control group, which did not receive *B. infantis* and were not administered DSS. Group 2 was the positive control group as these mice received DSS but not *B. infantis*. Groups 3 and 4 were both administered DSS and *B. infantis*, while group 4 was also injected i.p. with citral (as described above). Group 5 received DSS and citral. Mice were fed *B. infantis* for 7 days before colitis induction. Mice received DSS (TdB Consultancy AB, Uppsala) in water (2.5%) for 6 days followed by 2 days without DSS. During this period bacteria were administrated daily by gavage ($1x10^9$ cfu/mouse). All mice were euthanized on the final day of the study using cervical dislocation, which was performed by an experienced investigator.

Cell isolation

Single cell suspensions from mesenteric lymph nodes (MLN) and Peyer's patches (PP) were isolated using C tubes and GentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer instructions. LP cells were isolated from the upper part of small intestine (SI). A 5cm long piece of SI was washed out with cold calcium and magnesium free PBS (CMF-PBS) containing 1mmol dithiothreitol (DTT) and cold CMF-PBS containing 12 mmol EDTA. The SI was cut into pieces and vortexed in CMF-PBS containing 0.3 mmol EDTA. After centrifugation

(300g/5minutes) tissue was digested for 45minutes at 37C in RPMI containing 25kU/l collagenase IV (Sigma), 150mg/l DNase I (Roche, Rotkreuz, Switzerland) and 5% fetal calf serum (FCS, Sigma). Cell suspensions were filtered through 70µm cell strainers, centrifuged (700g/8minutes) and washed with CMF-PBS containing 5% FCS, 5mg/l DNase I, 5mmol/l EDTA. Finally pellets were resuspended in cRPMI (Invitrogen, LuBioScience, Luzern, Switzerland).

Flow cytometry and cell imaging

Anti-mouse CD11b, CD11c, MHCII, CD3, CD19 and CD103 antibodies (Biolegend, Lucerna-Chem, Luzerna, Switzerland) were used for characterization of dendritic cell phenotypes. RALDH activity was measured with the ALDEFLOUR kit (Aldagen, Durham, USA) according to manufacturer instructions. Anti-mouse CD3, CD4, CD25, LPAM-1 (integrin α4β7) and CCR9 antibodies (Biolegend) and anti-mouse Foxp3, IL-10, IL-4, IL-17A, IFN-γ antibodies (eBioscience San Diego, CA, USA) were used to characterize lymphocyte phenotypes. Cells for intracellular cytokine staining were pre-stimulated for 4 hours with PMA (50ng/ml, Sigma) and ionomycin (500ng/ml, Sigma) in the presence of Brefeldin A (1µg/ml, eBioscience). Flow cytometric analysis was performed using a 10 colour Galios flow cytometer (Beckman Coulter, Brea, USA). Kaluza (Beckman Coulter) was used for data analysis.

MLN and LP cells were incubated *in vitro* for 1 hour with CFSE labeled *B. infantis*. Cells were stained with anti-mouse CD11c and CD103. Bacteria binding was visualized using multispectral imaging flow cytometer Image Stream X (Amnis Corporation, Seattle, USA) and images were analyzed using IDEAS software (Amnis Corporation).

Histology

Colons were removed, flushed with PBS and wrapped around to generate a swiss roll. Swiss rolls were fixed in 4% paraformaldehye for 12 hours and stored in PBS until paraffin embedding. Following embedding, 3 µm thick sections were stained with Gill's hematoxylin and eosin (Sigma). Mounting was done with Eukitt ® quick-hardening mounting medium (Sigma). Tissue samples were analyzed by a pathologist in a blinded manner. The histology score included assessment of crypts dilatation, inflammatory cells infiltration in LP, inflammatory cells infiltration in submucosa, necrosis of

epithelium and submucosal edema. Each parameter was scored from 0 to 5 resulting in a maximum score of 25.

Myeloperoxidase (MPO) activity test

Pieces of colon were homogenized in 50mM potassium phosphate buffer, pH 6.0, containing 0.3% hexadecyltrimethyl-ammonium bromide (HTAB, Sigma). Tissue was sonicated on ice for 15 seconds followed by 3 freeze-thaw cycles. Samples were centrifuged and 20µl of the supernatant was mixed with 200µl freshly prepared 50mM potassium phosphate buffer, pH 6.0, containing 0.3% HTAB, O-dianisdine dihydrochloride (0.167mg/ml, Sigma) and 0.5% hydrogen peroxide (Sigma). Change in absorbance was measured at 450nm over 4 min. by an ELISA plate reader.

Cytokine assay

$1x10^6$ SI-LP cells were cultured in 1ml cRPMI containing amphotericin B (6,25µg/ml, Sigma) and gantamycin (12,5µg/ml, Sigma) and supernatants were collected after 24 hours. Cytokine secretion was examined by Bio-Plex multiplex suspension array (Bio-Rad Laboratories, Hercules, USA).

Statistics

Unpaired student t-tests were used to analyse data with a normal distribution, while the non-parametric Mann-Whitney test was used to analyze the non-parametric data. All data analysis was carried out using GraphPad Prism software. A p value of <0.05 was used as the cutoff for statistical significance.

RESULTS

B. infantis is sampled by Peyer's patch and lamina propria dendritic cells

Dendritic cells within the LP and Peyer's patches (PP) have been previously described to sample bacteria from the gastrointestinal lumen. In order to determine whether *B. infantis* was sampled by dendritic cells from either site, CFSE-labelled bacteria were gavaged to mice and single cell suspensions were generated from ileal LP and PP after 2 hours. Within the PP, $CD11c^{+}MHCII^{+}$ dendritic cells were identified, which had become CFSE positive 2 hours after feeding (Figure 1a). $CD11c^{+}MHCII^{+}$ dendritic cells within the LP also became CFSE positive at 2 hours and with higher frequency (Figure 1a). The presence of CFSE-labelled bacteria attached to, or internalized by, PP dendritic cells at 2 hours was confirmed using multispectral flow cytometry imaging (Figure 1b).

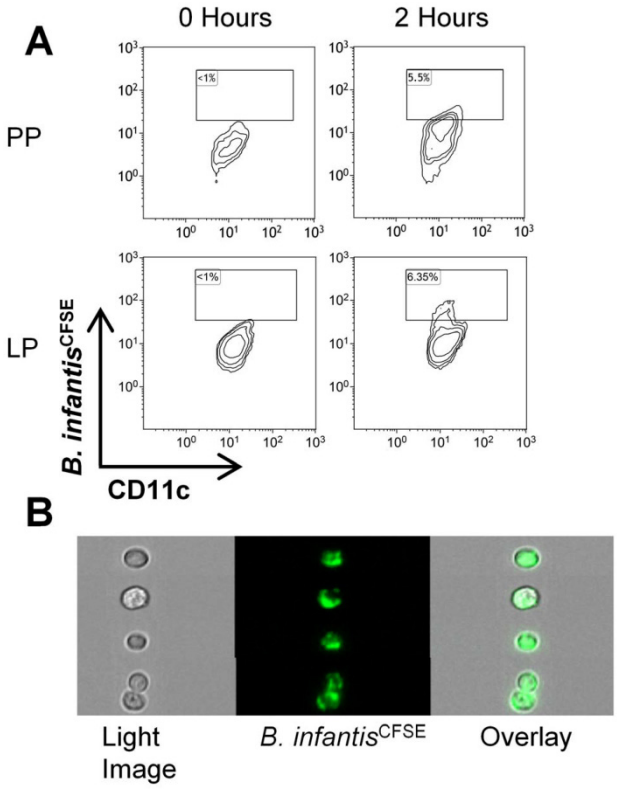


Figure 1. *B. infantis* is sampled by dendritic cells within PP and LP.

(a) Flow cytometric analysis of $CD11c^{+}MHCII^{+}$ dendritic cells within the PP and LP at 0 or 2 hours following gavage of CFSE-labelled *B. infantis*, revealed that a subpopulation of dendritic cells at both sites become $CFSE^{+}$. **(b)** Visualization by multispectral flow cytometry imaging confirmed the presence of CFSE-labelled bacteria on PP dendritic cells at 2 hours.

B. infantis induces CD103+ retinoic acid secreting dendritic cells

We examined CD103 expression and retinoic acid secretion by dendritic cells in mesenteric lymph nodes (MLN) and ileal LP following *B. infantis* feeding for 7 days. *B. infantis* feeding was associated with a significant increase in the percentage of dendritic cells that were $CD103^+$ and metabolizing retinoic acid in the LP, while the increase in retinoic acid metabolizing $CD103^+$ dendritic cells in the MLN approached statistical significance (Figure 2a). Citral treatment, which blocks retinoic acid metabolism, reduced the *B. infantis*-induced increase in $CD103^+$ and retinoic acid metabolizing dendritic cells within the LP. In order to determine if the increase in retinoic acid metabolism was a direct effect of *B. infantis* binding to $CD103^+$ dendritic cells, *in vitro* co-incubation with mucosal dendritic cells demonstrated that $CD103^+$ dendritic cells were able to bind *B. infantis* at a high frequency, while CD103- dendritic cells bound *B. infantis* at a low frequency (Figure 2b and Supplementary figure 1). Moreover, $CD103^+$ dendritic cells gene expression of the retinoic acid metabolizing enzymes *ALDH1a1* and *ALDH1a2* were significantly upregulated following co-incubation with *B. infantis* (Figure 2d). In addition, $CD103^+$ and $CD103^-$ dendritic cells were isolated from the mucosa by flow cytometric sorting and were co-incubated with *B. infantis*. Gene expression for *ALDH1a1* and *ALDH1a2* was significantly increased following *B. infantis* co-incubation only in $CD103^+$ dendritic cells, but not $CD103^-$ dendritic cells (Figure 2e).

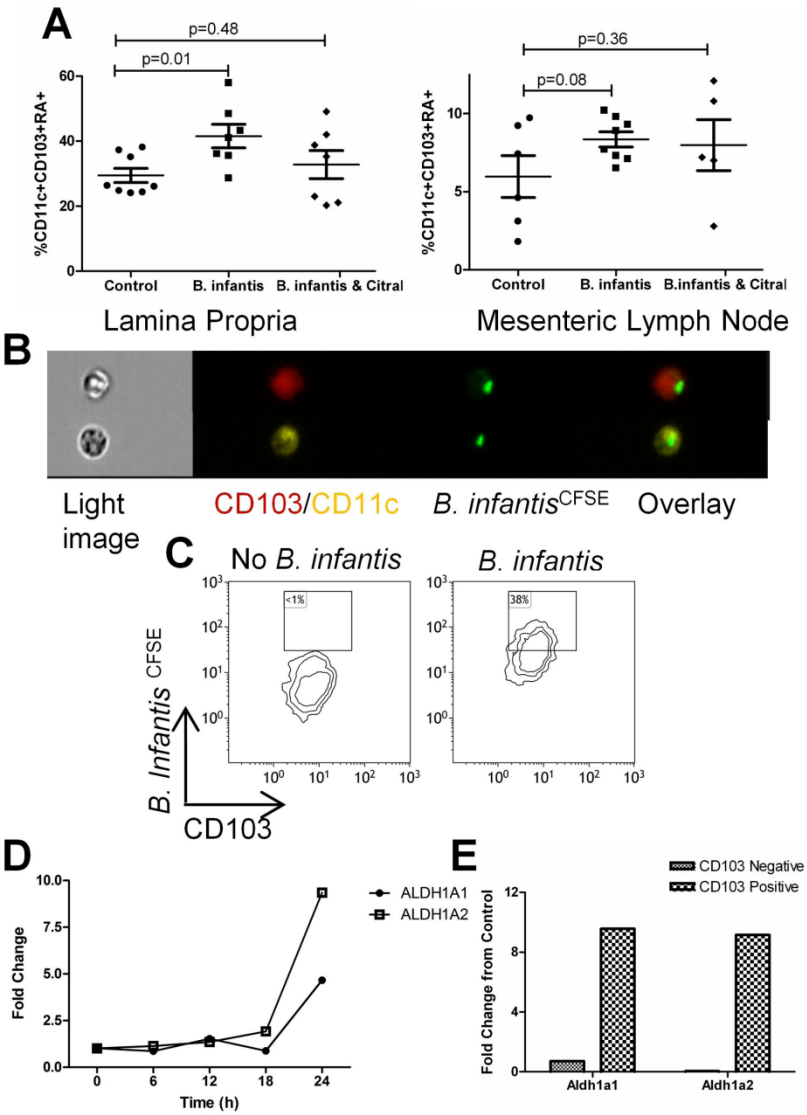


Figure 2. CD103^{+}RALDH^{+} dendritic cells are elevated in the LP following *B. infantis* feeding.

Flow cytometric assessment of LP and MLN revealed that *B. infantis* feeding is associated with increased CD103^{+}RALDH^{+} dendritic cells within the LP (n=7), compared to the control group (n=8), analysed using unpaired student t-tests **(a)**. Citral blocked the increase in LP CD103^{+}RALDH^{+} dendritic cells (n=7). **(b)** Multispectral flow cytometry imaging identified CD103^{+} dendritic cells that efficiently bind CFSE-labelled *B. infantis*. **(c)** Flow cytometric analysis of CD11c^{+}MHCII^{+}CD103^{+} dendritic cells from the mucosa demonstrated that approximately 38% of CD103^{+} dendritic cells bound *B. infantis*. Isolated mucosal CD11c^{+} dendritic cells upregulate mRNA for RALDH enzymes following *in vitro* incubation with *B. infantis* **(d)**, while the increase in gene expression is specific to CD103^{+} dendritic cells **(e)**.

B. infantis suppression of T_H17 cells is retinoic acid-dependent

Ileal LP single cell suspensions were examined for IL-17, IFN-γ and IL-4 positive lymphocytes. The percentage of IL-17^+ lymphocytes within the LP was significantly reduced following *B. infantis* feeding for 7 days, while the reduction in IFN-γ^+ lymphocytes approached statistical significance (Figure 3a). Citral treatment reversed the *B. infantis* suppression of IL-17^+ and IFN-γ^+ lymphocytes within the LP. Neither *B. infantis* feeding nor citral treatment altered the percentage of IL-4^+ lymphocytes within the LP (Figure 3a). Isolated LP cells were cultured *in vitro* for 24 hours and spontaneous secretion of T_H17 polarizing cytokines were measured in culture supernatants. Both IL-1β and IL-6 secretion were significantly reduced by *B. infantis* feeding. Citral reversed the suppression of IL-1β secretion and partially reversed the suppression of IL-6 secretion (Figure 3b). One potential explanation for the *B. infantis* suppression of T_H17 cells within the LP is that *B. infantis* may alter lymphocyte recruitment to the LP. Expression of the intergrin α4β7 and the chemokine receptor CCR9 influence the gut homing of lymphocytes. Both $\alpha4\beta7^+$ and $CCR9^+$ lymphocytes populations were significantly suppressed following *B. infantis* feeding (Figure 3c). However, citral treatment had no effect on the *B. infantis* suppression of $\alpha4\beta7^+$ lymphocytes, with a minor effect on $CCR9^+$ lymphocytes, suggesting that the *B. infantis* effect on lymphocyte homing is independent of retinoic acid metabolism.

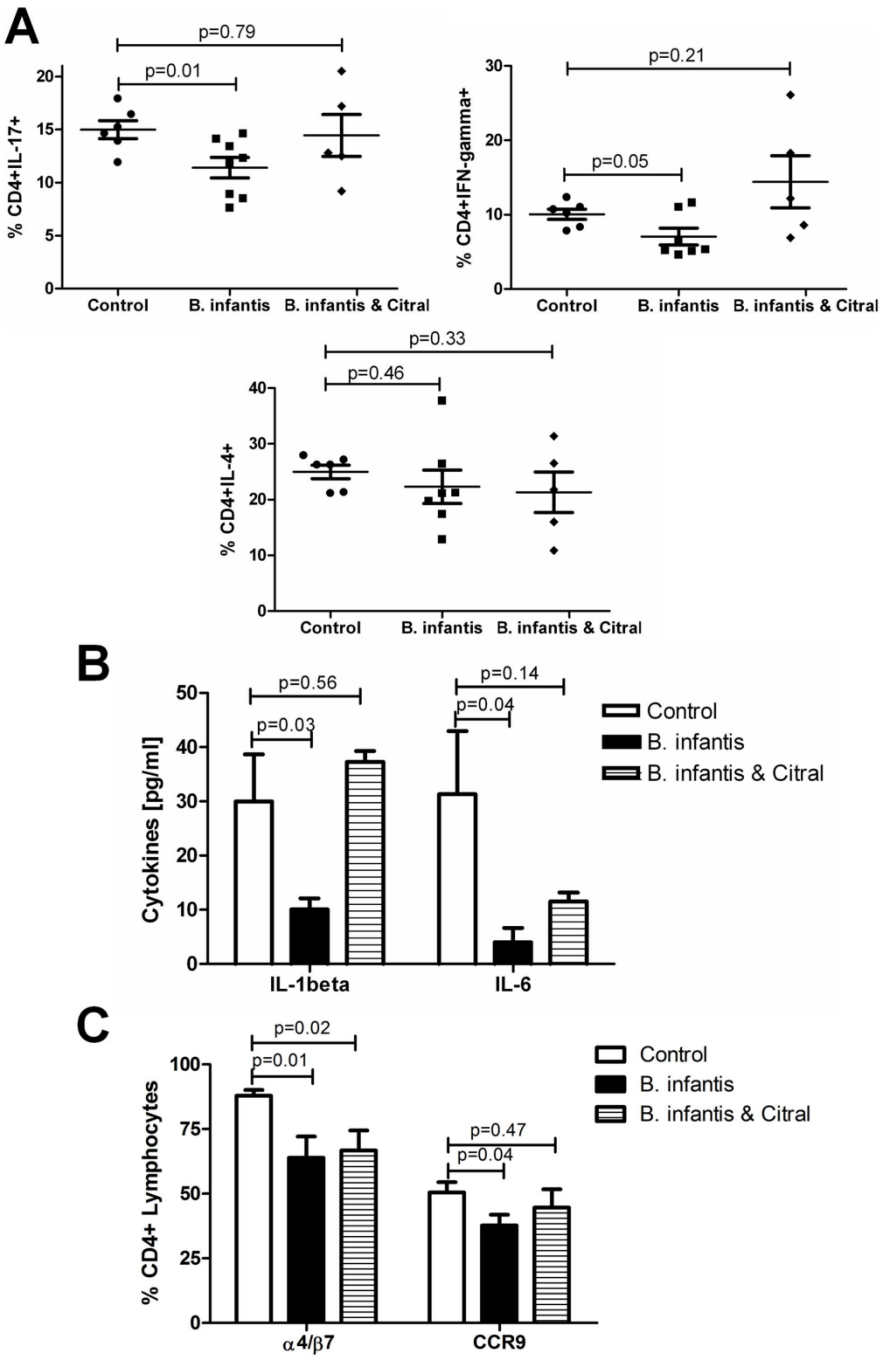


Figure 3. *B. infantis* alters lymphocyte phenotypes within the LP.

(a) IL-17$^+$ lymphocytes were significantly reduced and IFN-γ$^+$ cells were substantially reduced within the LP of *B. infantis*-fed mice (n=8), compared to the control group (n=6), an effect that was blocked by citral (n=5). No effect was observed for IL-4$^+$ lymphocytes. **(b)** Isolated LP was cultured *in vitro* and cytokine secretion measured after 24 hours. *B. infantis* feeding reduced the *in vitro* secretion of the T_H17-polarising cytokines IL-1β and IL-6, which was partially reversed by citral. **(c)** *B. infantis* feeding was associated with a decrease in the proportion of LP lymphocytes expressing the gut homing receptors α4β and CCR9. Citral did not reverse the decrease in α4β7$^+$ lymphocytes and had a minor influence on CCR9$^+$ lymphocytes. Statisical significance was estimated using unpaired student t-tests.

B. infantis induction of Foxp3$^+$ lymphocytes is retinoic acid-independent

As previously described, *B. infantis* feeding was associated with increased numbers of regulatory lymphocytes within the mucosa (Figure 4a). Foxp3$^+$ T lymphocytes were increased within the LP and showed a tendency to be increased for MLN (Figure 4b). However, citral did not attenuate the increase in Foxp3$^+$ lymphocytes suggesting that mechanisms other than retinoic acid metabolism can be responsible for this effect. IL-10$^+$ CD4$^+$lymphocytes were significantly increased in the MLN, but not the LP of *B. infantis*-fed animals, which was blocked by citral (Figure 4c).

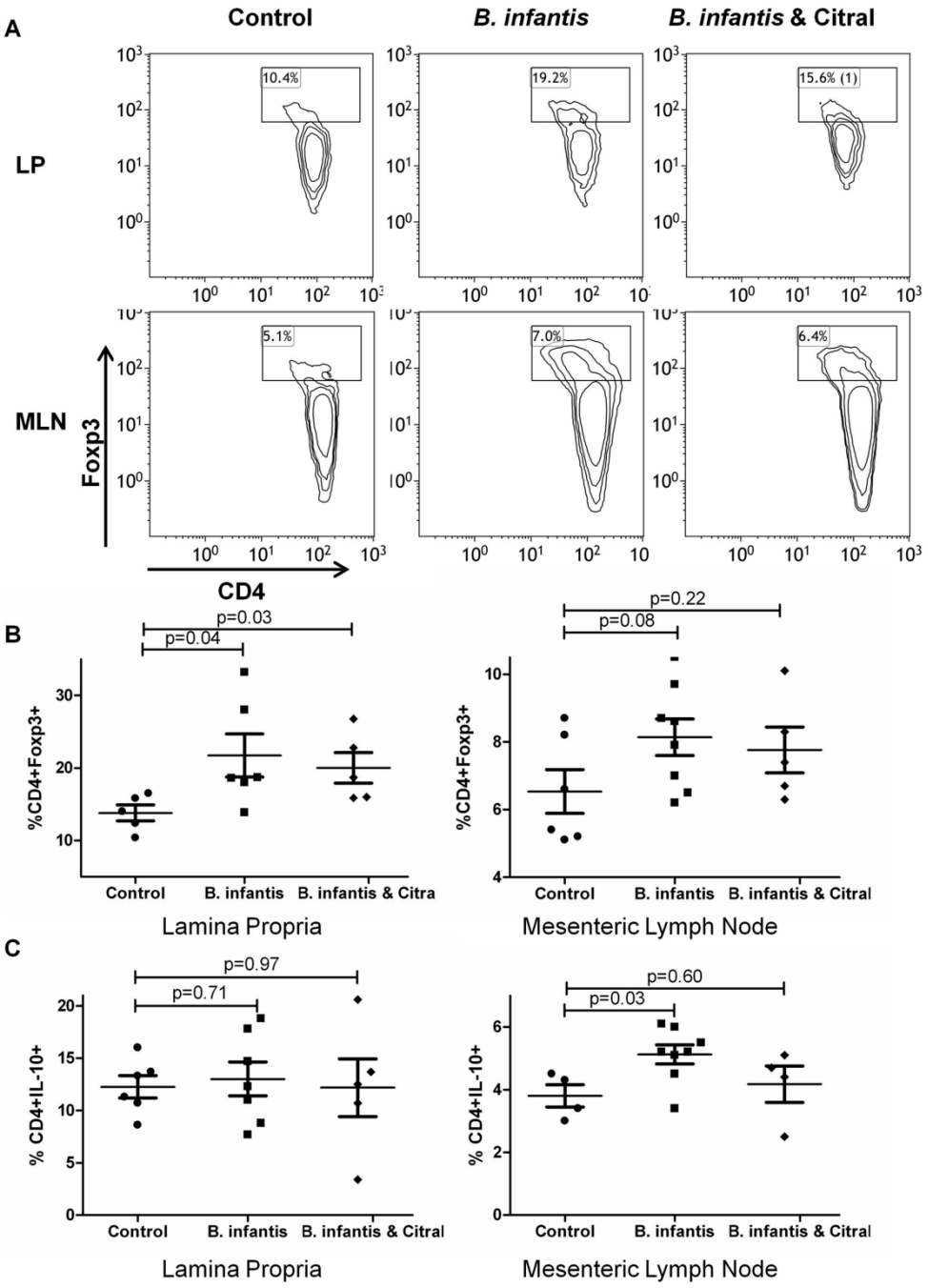


Figure 4. *B. infantis* induction of Foxp3$^+$ lymphocytes is not RALDH-dependent.

(a) Representative flow cytometric dot-plots are illustrated for CD4 and Foxp3 populations within MLN and LP. **(b)** The increase in CD4$^+$Foxp3$^+$ T lymphocytes following *B. infantis* feeding in the LP (n=6) is not reversed by citral treatment (n=5). **(c)** CD4$^+$IL-10$^+$ T lymphocytes increased within MLN (n=8), but not the LP (n=7). LP statistical significance was estimated using the non-parametric Mann-Whitney test, while MLN statistics were determined using the parametric unpaired student t-test.

B. infantis attenuation of colitis is retinoic acid-dependent

In the DSS model of colitis, *B. infantis* feeding was associated with decreased histopathology (Figure 5a and 5b) and decreased MPO levels (Figure 5c). Citral treatment blocked the protective effect of *B. infantis* feeding. Citral itself did not impact the severity of DSS-induced colitis (Figure 5a) or LP T_H1 and T_H17 subsets (Figure 6a). However, *B. infantis* significantly reduced the numbers of IL-17$^+$ and IFN-γ^+ lymphocytes within the LP of colitic animals, which was blocked by citral treatment (Figure 6a). The proportion of Foxp3$^+$ lymphocytes within the MLN increased significantly due to the induction of colitis and *B. infantis* feeding had no effect on the increase in Foxp3$^+$ lymphocytes within the MLN (Figure 6b). In contrast, DSS-induced colitis was associated with a significantly reduced percentage of Foxp3$^+$ lymphocytes within the LP and *B. infantis* feeding partially reversed the drop in Foxp3$^+$ lymphocytes within the LP (Figure 6b). As described above, *B. infantis* feeding to healthy animals significantly reduced $\alpha4\beta7^+$ and CCR9$^+$ lymphocytes within the LP, however *B. infantis* feeding to animals with DSS-induced colitis did not alter $\alpha4\beta7^+$ or CCR9$^+$ lymphocyte numbers (Figure 6c). Colitis was associated with significantly increased numbers of CD103$^+$ dendritic cells within the LP (Figure 7a). However, the percentage of CD103$^+$ dendritic cells that were RALDH$^+$ were significantly reduced in the control DSS group, while *B. infantis* feeding reversed the suppression of RALDH$^+$ CD103$^+$ dendritic cells (Figure 7b). Moreover, the frequency of CD11c$^+$ CD103$^-$RALDH$^+$ cells was reduced in the inflamed LP and *B. infantis* feeding did not affect retinoic acid production by CD103$^-$ cells (Figure 7c). The upregulation of CD103 in the colitis group was primarily seen on CD11c$^+$CD11b$^+$ dendritic cells, while the upregulation of CD103 in the *B. infantis* group was within the CD11c$^+$CD11b$^-$ dendritic cell subpopulation (Figure 7d).

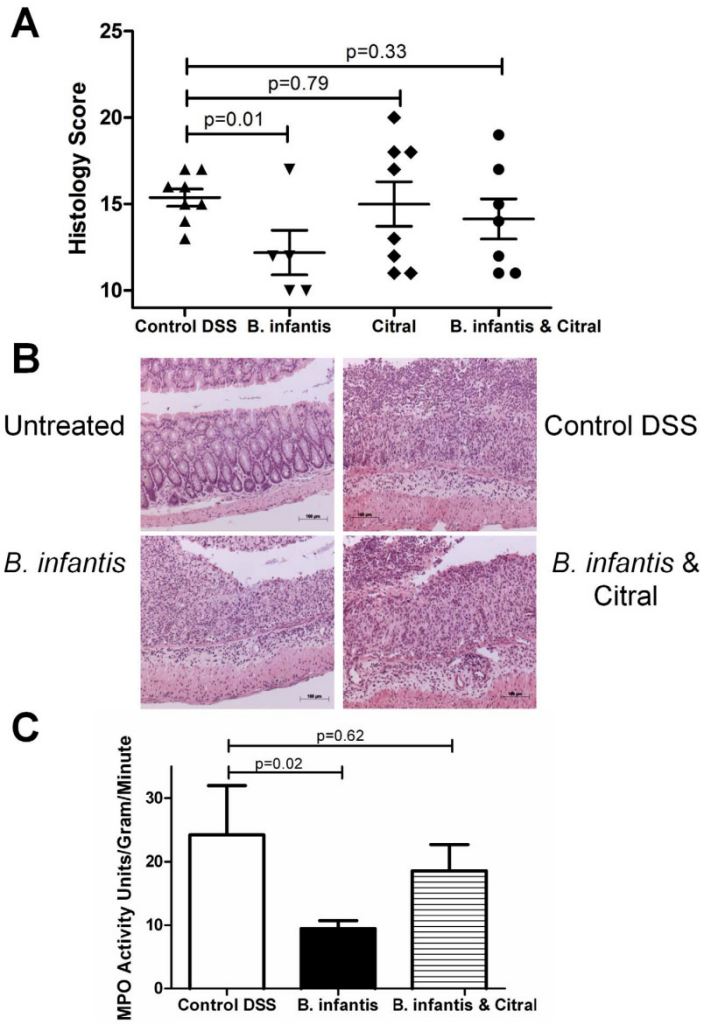


Figure 5. DSS-induced colitis is reduced by *B. infantis* feeding.

Four murine study groups were examined – Untreated (no DSS and no *B. infantis*), Control (DSS alone), *B. infantis* (DSS and *B. infantis*), citral (DSS and citral) and *B. infantis* & citral (DSS, *B. infantis* and citral). **(a)** The histopathology inflammatory score was significantly reduced by *B. infantis* feeding (n=5), but not when citral was co-administered (n=8). Citral administered with DSS did not increase inflammation in the colon in comparison to DSS alone (n=7). **(b)** Representative slides of the murine gut are illustrated. **(c)** Colonic myeloperoxidase (MPO) levels were reduced in *B. infantis*-fed mice, which was not observed with *B. infantis* and citral treatment. Statistical significance was determined using non-parametric Mann-Whitney tests.

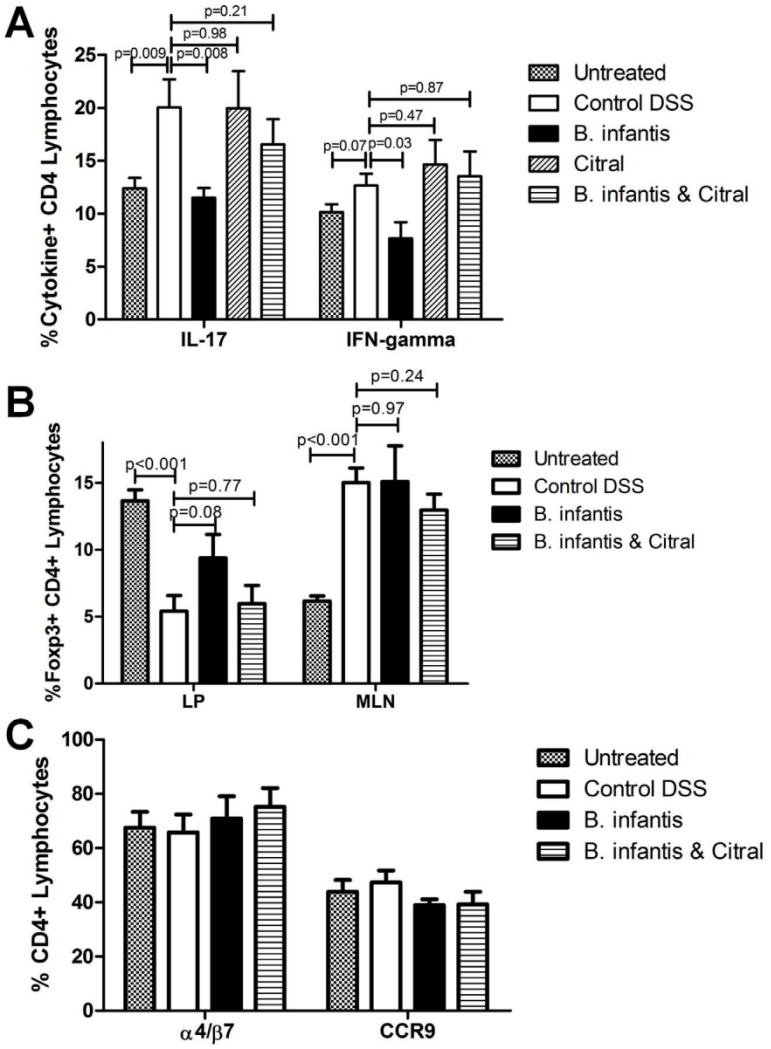


Figure 6. *B. infantis* alters T cell phenotypes within the inflamed LP.

(a) DSS colitis increases the proportion of IL-17$^+$ and IFN-γ$^+$ CD4 T lymphocytes within the LP (n=8). However, *B. infantis* feeding (n=6) significantly reduces LP IL-17$^+$ and IFN-γ$^+$ subpopulations compared to DSS alone, which was inhibited by citral (n=7). Both subpopulations were similarly increased in DSS alone and DSS & citral (n=7). **(b)** CD4$^+$Foxp3$^+$ lymphocytes were significantly increased in the MLN during DSS colitis (n=6), with a significant decrease of Foxp3$^+$ lymphocytes being observed within the LP (n=6). *B. infantis* feeding (n=6) did not alter the increase in Foxp3$^+$ cells in the MLN, but partially restored the deficit in Foxp3$^+$ cells within the LP (n=6). **(c)** Expression of the gut homing receptors α4β7 and CCR9 did not significantly change for any of the groups examined. Statistical significance was determined using unpaired student t-tests.

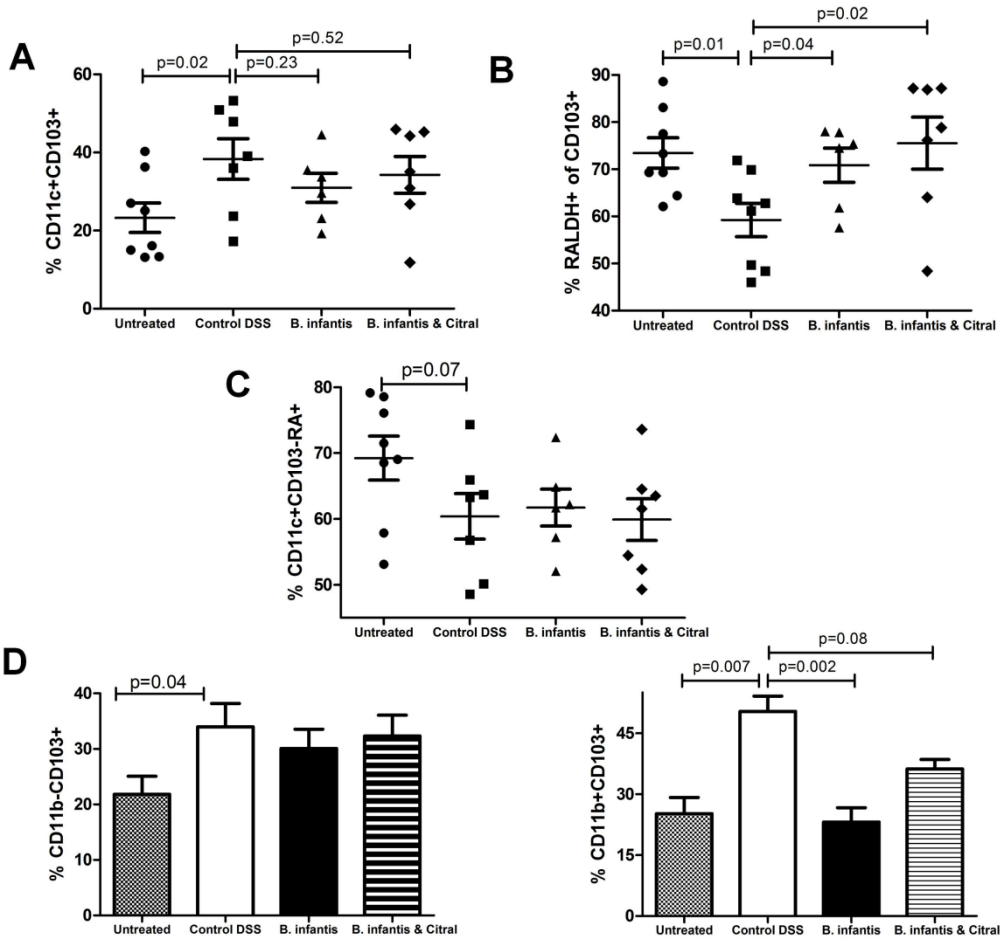


Figure 7. *B. infantis* suppresses the expansion of a pro-inflammatory dendritic cell phenotype within the LP.

Untreated (n=8), DSS colitis (n=7), DSS and *B. infantis* treated (n=6) and DSS, *B. infantis* and citral treated animals (n=7) were compared for dendritic cell subsets within the LP. **(a)** The proportion of $CD103^+$ dendritic cells within the LP was increased for all groups with DSS-induced colitis. **(b)** However, the $CD103^+$ cells expressing RALDH was significantly reduced in the inflamed LP, which was normalized by *B. infantis* feeding. **(c)** The $CD11c^+CD103^-RALDH^+$ cell numbers were reduced in the control DSS group and *B. infantis* did not reverse this suppression. **(d)** The increase in $CD103^+$ dendritic cells in the inflamed LP primarily consists of $CD11b^+CD103^+$ dendritic cells, while *B. infantis* feeding was associated with an increase in $CD11b^-CD103^+$ dendritic cells. Statistical significance for (a), (b) and (c) was estimated using the non-parametric Mann-Whitney test, while (d) statistics were determined using the parametric unpaired student t-test.

DISCUSSION

In this study, we show that *B. infantis* is sampled by mucosal dendritic cells within the LP and PP, resulting in increased numbers of LP $CD103^+RALDH^+$ dendritic cells with tolerogenic properties. The suppression of T_H1 and T_H17 lymphocytes within the LP was observed in the healthy and inflamed gut, which was dependent on retinoic acid metabolism as citral administration blocked this activity. In addition, disease severity was reduced by *B. infantis* feeding during DSS-induced colitis, which was also blocked by citral. Interestingly the elevation in mucosal LP Foxp3 lymphocytes, but not MLN IL-10^+ lymphocytes, associated with *B. infantis* feeding was RALDH-independent.

LP $CD103^+$ dendritic cells are derived from circulating common dendritic cell precursors (not from LP $CD103^-$ intermediates), require Flt3 ligand for their development and migrate efficiently to the draining lymph nodes [22, 23]. However, the microbial factors that influence the tolerogenic potency of $CD103^+$ dendritic cells within the LP are only beginning to be elucidated. One study using germ-free animals suggested that $CD103^+$ dendritic cells within the colon did not require the presence of a microbiota [24]. In contrast, another study recently demonstrated that *Bifidobacterium breve* promoted development of IL-10-producing Tr1 cells in the colon by intestinal $CD103^+$ dendritic cells via the TLR2/MyD88-dependent induction of IL-27 and IL-10 [25]. We have also previously demonstrated *in vitro* that *B. infantis*-induced IL-10 secretion by human myeloid dendritic cells was TLR2-dependent [21]. However, Jeon et al did not detect alterations in T_H17 or T_H1 populations within the colon following *B. breve* feeding [25]. As they did not measure $CD103^+RALDH$ activity, direct comparisons with the present study are not possible but it's clear that the *B. infantis*-associated suppression of T_H17 and T_H1 cells is dependent on RALDH activity.

Intestinal homeostasis is maintained by regulatory T cell populations consisting of two major $CD4^+$ T cell subsets, $Foxp3^+$ T_{reg} cells and IL-10-producing Tr1 cells [26]. Site-specific alterations in regulatory lymphocyte subsets are evident in this study. Within the LP, *B. infantis* increased the proportion of $Foxp3^+$ lymphocytes, while Tr1 cells were increased only within the MLN. Alternative mechanisms also are required for induction of the two regulatory populations as citral blocked the induction of MLN Tr1 cells, but not the elevation in $Foxp3^+$ cells within the LP. Suppression of T_H1 and T_H17 lymphocytes within the LP was abrogated by citral confirming that citral administration did have an effect within the LP. Thus, the *B. infantis* induction of

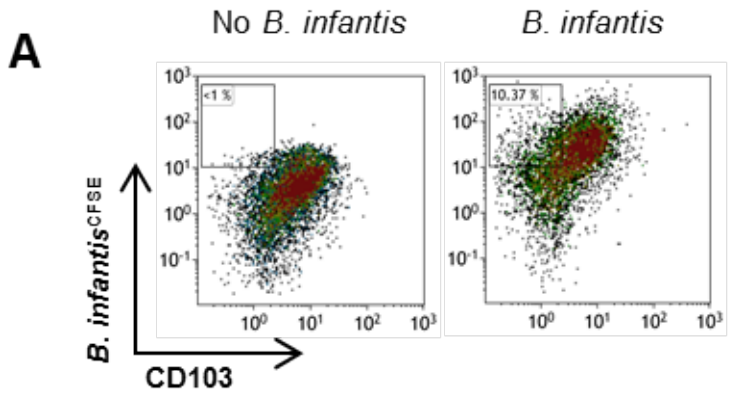
Foxp3$^+$ lymphocytes within the LP involves mechanisms other than retinoic acid metabolism. Similarly, *B. infantis* reduced the proportion of α4β7 and CCR9 lymphocytes within the LP, which was not dramatically influenced by citral. Retinoic acid has been previously described to upregulate expression of gut homing receptors, which was not observed in our studies. This finding further supports the existence of additional non-RALDH-dependent mechanisms which are induced by *B. infantis* within the LP [27].

Even though RALDH and non-RALDH mechanisms may be required for *B. infantis*-associated immunoregulatory activity within the mucosa, citral blocked the anti-inflammatory effect of *B. infantis* in the DSS colitis model confirming that the induction of retinoic acid metabolism is critical for the *in vivo* protective effects of this microbe. Similar to the findings with healthy mice, *B. infantis* reduced T_H1 and T_H17 lymphocytes within the inflamed LP, which was retinoic acid-dependent. In contrast to healthy mice, *B. infantis* did not reduce the number of lymphocytes expressing α4β7 and CCR9 within the inflamed LP. While certain therapeutic approaches have focused on blocking gut homing receptors for amelioration of colitis, it has also been shown that CCR9 deficiency exacerbates colitis due to impairment of T_{reg} recruitment to the gut [28, 29]. However, our study suggests that *B. infantis* does not alter recruitment of α4β7 or CCR9 positive lymphocytes into the inflamed LP.

During DSS-induced colitis, the relative proportion of CD11b$^+$CD103$^+$ dendritic cells was increased within the LP, while *B. infantis* feeding was associated with an increase in CD11b$^-$CD103$^+$ dendritic cells. The LP CD11b$^+$CD103$^+$ dendritic cell subset has been suggested to possess proinflammatory properties within the inflamed gut [11, 30]. In addition, this dendritic cell population was shown to play key role in T_H17 cell differentiation *in vitro* [31]. Furthermore, CD11b$^+$CD103$^+$ dendritic cells express TLR5 at a high level, rapidly respond to flagellin stimulation resulting in IL-23 secretion and are very efficient in presenting antigens to CD4+ lymphocytes [12, 32, 33]. Our data suggests that *B. infantis*, even within an inflamed microenvironment, continues to induce regulatory CD11b$^-$CD103$^+$RALDH$^+$ dendritic cells within the LP and suppresses the increase in the proinflammatory CD11b$^+$CD103$^+$ dendritic cell population. Interestingly, the suppression of T_H17 cells within the inflamed LP of *B. infantis*-fed mice correlates with the suppression of the CD11b$^+$CD103$^+$ dendritic cell subset. Further investigation is required to determine if there is a direct connection between *B. infantis* associated suppression of CD11b$^+$CD103$^+$ dendritic cells and T_H17 polarisation.

Within the mucosa, dendritic cells are integral to promoting oral tolerance and preventing pathological immune responses to harmless antigens. Dendritic cells use signals derived from their local environment to shape regulatory and low-level immune responses to the commensal microbiota, which controls the microbiota without causing pathology. The breakdown in dendritic cell regulatory networks is associated with aberrant inflammatory activity within the gut and therapeutic strategies aimed at re-establishing dendritic cell tolerogenic tone would be of benefit to IBD, IBS and food allergy patients. One such strategy is the deliberate manipulation of $CD103^{+}RALDH^{+}$ dendritic cells by microbes or microbial components in combination with dietary supplementation with vitamin A. The murine data presented in this report strongly support the further evaluation of these strategies in human clinical studies.

SUPPLEMENTARY DATA



Supplementary Figure 1. B. infantis is bound at a low frequency by CD11c^{+}MHCII^{+}CD103^{-} dendritic cells.

Flow cytometric analysis of CD11c^{+}MHCII^{+}CD103^{-} dendritic cells from the mucosa demonstrated that approximately 10% of CD103^{-} dendritic cells bound *B. infantis*.

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8. Final discussion

Mucosal immune tolerance is a dynamically developing field of immunology. On our mucosal surfaces we have contact with commensal microbes, pathogens and allergens. DCs sample this environment and shape subsequent immune responses creating a balance between tolerance and inflammation. Induction of Foxp3 T regulatory cells and the molecular mechanisms involved in this process are being deeply investigated.

B. infantis 35624 was shown to protect against inflammation in many murine models, such as colitis and allergic inflammation. The cellular mechanism of action included Foxp3 T regulatory cells but the molecular mechanisms were unknown. Moreover, human trials with this bacterium were performed. For instance, oral consumption of *B. infantis* led to symptoms improvement in patients with Irritable Bowel Syndrome (31, 32). Recently, the concept of a central tolerogenic mechanism driven by specific gut microbes was supported in a human trial with *B. infantis*. Oral consumption of this bacterium attenuated inflammatory markers, not only in patients with gut inflammation, but also in other disorders such as chronic fatigue syndrome and psoriasis (33). This may be explained by an increase in Foxp3+ Tregs in the peripheral blood, which was at least demonstrated for healthy volunteers fed with *B. infantis*. Tregs could reduce inflammation at different sites, leading to a decrease in pro-inflammatory cytokines, such as TNF-α in patients plasma. As IL-6 and TNF-α are positively correlated with C reactive protein (CRP) production, their reduction may result in downregulation of CRP production by the liver and adipose tissue. Moreover, induction of vitamin A metabolism in the gut may result in higher retinoic acid concentration in the plasma and subsequent anti-inflammatory effects in other tissues.

8.1. *B. infantis*-dependent increase in Foxp3+ Treg cells

In chapter 7.1 we demonstrated that oral feeding of healthy human volunteers with *B. infantis* leads to an increase in CD4+Foxp3+ T cells within peripheral blood, which is consistent with observations from murine models. In addition, the Foxp3 population displayed enhanced expression of inducible T-cell costimulator (ICOS) and cytotoxic T-lymphocyte antigen 4 (CTLA-4), suggesting that these cells are functional Tregs (263). Numbers of Foxp3+ cells in CD25+ population were increased, while no Foxp3 changes were observed for CD25- T cells. This effect can be related to the promotion of CD25 expression (264) or to stabilization and expansion of already existing CD4+CD25+Foxp3+ Treg cells. In a murine model of Salmonell infection, inflammation was attenuated by *B. infantis* induced Treg cells (27). NF-κB activation, proinflammatory cytokine secretion and tissue damage were reduced in a Treg dependent manner. This ability of specific microbes to induce and/or promote Treg cells development may be restricted for certain bacterial strains within the gut microbiota. In fact, studies with other bacteria: *Bifidobacterium longum* AH1206, *Bifidobacterium breve* AH1205 and *Lactobacillus salivarius* AH102 demonstrated that only AH1206 induced Treg cells and protected against respiratory allergic inflammation (28). Moreover, we have shown a role for DCs in Foxp3 cells induction. In *in vitro* experiments both mDCs and pDCs, isolated from human peripheral blood, respond to *B. infantis* but via different PRRs. Foxp3 induction by mDCs and MDDCs requires TLR-2, DC-SIGN and retinoic acid metabolism, while pDCs require IDO activation. This was the first report, which suggested that a single commensal bacterial strain activates distinct molecular pathways in different human DC subsets, leading in to the same result – Foxp3 enhancement in CD4 T cells.

8.2. Activation of TLR2 and DC-SIGN signaling pathways

Both DC subsets: mDCs and pDCs secrete IL-10 but not IL-12 in response to *B. infantis* stimulation. Moreover, pDCs did not upregulate IFN-α gene expression, whereas control cells stimulated with CpG DNA expressed IFN-α. By use of blocking ODNs we demonstrated that *B. infantis* activates TLR9 in pDCs. This suggests that other receptors on pDCs have to be involved in *B. infantis* recognition and modulate cytokine secretion. For instance, in case of pDCs BDCA-2 is well described as a receptor, which can decrease TLR9 induced IFN-α secretion (154). mDCs and MDDCs recognize *B. infantis* via TLR2 and more precisely via the TLR2/6 heterodimer. This receptor was shown to be critical for immune response to microbes. TLR2-/- mice are more susceptible to DSS- induced colitis and TLR2 is involved in promoting Foxp3 expression in response to intestinal microbes (97, 98). In humans, TLR2 SNPs were associated with disease phenotype in IBS patients. Interestingly, we could observe two classes of IL-10 secretion in response to *B. infantis*. One group responded with very high level of IL-10 secretion, while the second group with low level secretion. TLR2 blockade led to a stronger decrease in IL-10 secretion in the first group. This effect could possibly be related to TLR2 SNPs. Moreover, TLR2 blockade led to increased IL-12 and TNF-α expression. However, the link between TLR2 activation and suppression of IL-12 is unknown and could be directly or indirectly mediated. Tyrosine kinase Syk is recruited by TLRs upon ligand binding and mediates non-canonical NF-κB signaling pathway activation. This leads to IL-1β and IL-12p40 downregulation in a RELB-dependent manner (145). Moreover, Syk can interact with TRAF6 during MyD88-dependent TLR signaling and decrease pro-inflammatory cytokine production (265). Both Syk and ERK activation can be involved in IL-10 and RALDH2 upregulation, which leads to retinoic acid and IL-10 production. These anti-inflammatory factors are known to induce suppressor of cytokine signaling 3 (SOCS3) expression, which suppresses p38 MAPK and pro-inflammatory cytokines production, including IL-12, IL-6, TNF-α and IL-23 (224). Another possibility is that TLR2 via ERK mediates c-FOS phosphorylation and stabilization (266, 267). Subsequently, c-FOS represses IL-12 production, while inducing IL-10 production. All these pathways could potentially be responsible for direct and indirect IL-12 downregulation mediated by TLR2 (268). Moreover, TLR2 signaling can be modulated on different levels by numerous miRNA. DC-SIGN was also activated on MDDCs and this receptor blockade

led to increased TNF-α and reduced IL-10 secretion. DC-SIGN can influence TLR signaling by Raf-1 activation and subsequent acetylation of phosphorylated NF-κB subunit p65 via histone acetyltransferases CREB-binding protein (CBP). This results in enhanced IL-10 secretion (269). However, intermediate steps of this pathway remain unknown.

8.3. Retinoic acid metabolism and CD103+ DCs in the gut

It is well described that retinoic acid metabolism in intestinal cells and certain DC subsets promotes conversion of naïve T cells into Foxp3+ Tregs (270). In *in vitro* experiments, we observed upregulation of retinoic acid metabolizing enzyme RALDH2 in mDCs and MDDCs after stimulation with *B. infantis*. Activation of this metabolic pathway was critical for Treg induction in co-cultures with autologous CD4 T lymphocytes. Both TLR2 and DC-SIGN activation were important for RALDH2 upregulation and RALDH activity. TLRs regulation of retinoic acid metabolism has been partially described (195, 207, 223). Albeit, the signaling cascade leading to retinoic acid metabolism is unknown. There is no evidence suggesting how CLRs (such as DC-SIGN) can affect vitamin A metabolism and this topic needs further investigation. In contrast, bacterial stimulation of pDCs led to IDO upregulation (not RALDH) and use of 1MT (IDO activity inhibitor) blocked Treg development in co-cultures with autologous CD4 T cells.

Retinoic acid metabolism in the small intestine is well characterized but regulation by microbial stimuli is still not well described. CD103+ DCs are described as one of the main retinoic acid producers in the gut. They express Aldh1a2, which encodes the RALDH2 enzyme. In chapter 7.2 we demonstrated that *B. infantis* feeding induces retinoic acid metabolism significantly in LP CD103+ DCs. These DCs are derived from circulating common dendritic cell precursors, require Flt3 ligand for development and migrate to MLN (208, 271). They are still present in the colon of germ free mice (272), which suggests that the colonic population depends on factors other than the microbiota. In fact, retinoic acid metabolism in CD103+ DCs is increased after vitamin A administration and this effect is mainly present in the small intestine, where dietary vitamin A is easily accessible.

8.4. Bacteria sampling by CD103+ DCs in the gut

Sorted CD103+ DCs from MLN and PP were able to increase Aldh1a1 and Aldh1a2 expression after *B. infantis* stimulation, whereas CD103- DCs were not able to undergo these changes. Both CD103+ and CD103- DCs are involved in Treg priming. However, CD103+ have enhanced capability to produce retinoic acid in response to certain stimuli, such as TLR2 ligands. CD103+ DCs are more efficient in the induction of Foxp3 Treg differentiation than CD103- DCs and increase gut homing receptor expression on activated T cells (189). 38% of sorted CD103+ DCs from intestinal mucosa bound *B. infantis in vitro*. This suggests a specific role for intestinal CD103+ DCs in mediating *B. infantis*-mediated effects. There has been some controversy regarding the potential for DCs to sample commensal bacteria in the gut. Many groups have shown that pathogens, such as *Salmonella* are effectively sampled from the intestine lumen by CX3C chemokine receptor 1 (CX3CR1) or CD11c DC trans-epithelial dendrites (273, 274) but it was not clear if the same mechanisms apply for commensal microbes and if CD103+ DCs are also involved in this process. To address this question we administered carboxyfluorescein succinimidyl ester (CFSE)-labeled *B. infantis* by oral gavage and visualized bacteria binding to DCs in the PP and LP after 2 hours and 4 hours. We were able to show that more small intestinal LP DCs were CFSE positive after 4 hours, compared to 2 hours, whereas the number of PP CFSE DCs harboring bacteria was lower after 4 hours than after 2 hours. Recently, even more detailed investigation of bacteria binding by intestinal DC subsets was performed. Using 2-photon microscopy small intestinal CD103+ DCs, expressing tight junction proteins Claudin-4 and ZO-2, were shown to capture bacteria via their ability to form trans-epithelial dendrites (275). In steady-state CD103+ DCs are mainly located in murine LP. However, some of them patrol epithelium in the jejunum but not in the terminal ileum (274, 275). Some DCs are also found in the close proximity to murine colon epithelium but at lower level than in the murine small intestine (276). The transcytosis of bacteria via M cells into PP (277) seems to be more rapid and efficient than bacteria uptake by intestinal DCs monitoring gut lumen in steady-state. This could be a possible explanation for different dynamics of bacteria sampling, which we observed between small intestine LP and PP in our study with CFSE labeled *B. infantis*. It is worth mentioning that more CD103+ DCs forming dendrites were observed during infection with enterogenic pathogens, such as *Salmonella typhimurium* (275). In the small and

large intestine DCs were recruited to epithelium by chemokines secreted by epithelium and sentinel DC after first contact with pathogen (275, 278). However, in the small intestine this process was CCL-20 and CD103 independent (275), while in the colon CCL-5 and CCL-20 played important roles (278). Interestingly, intraepithelial CD103+ DCs sampling *Salmonella* in the murine small intestine were shown to harbor an additional marker CD11b (229). As CD103+CD11b+ DCs are highly pro-inflammatory, it opens the possibility for differential uptake of commensal and pathogenic bacteria by diverse CD103+ DC subsets in the lamina propria. There is not too much known about bacteria sampling in the human gut. However, the presence of CD103+ DCs in MLN was confirmed and potentially these cells can be also responsible for bacteria uptake in human intestine.

8.5. CD11b+CD103+ DCs in the small intestine

LP and MLN CD103+ RALDH+ DC numbers were increased in healthy animals after *B. infantis* feeding. This confirms that specific microbes can regulate retinoic metabolism *in vivo*. However, intestinal CD103+ DCs were shown to lose their tolerogenic properties in the presence of inflammatory mediators (279). This suggests that CD103 expression is not sufficient for identifying tolerogenic DCs under all conditions in the gut. Recently other markers were characterized on the surface of intestinal CD103+ DCs suggesting that this population can be further divided into smaller subsets. CD137 was proposed to be expressed by tolerogenic CD103+ DCs (280). In contrast, CD11b+CD103+DCs in the LP of the small intestine acquire proinflammatory properties under inflammatory conditions (281). They express TLR5 at a high level (257) and rapidly respond to flagellin stimulation with IL-23 secretion (282). Moreover, LP CD11b+CX3CR1-CD103+ DCs are very efficient in presenting antigens to CD4+ lymphocytes (283). During DSS-induced colitis, the relative proportion of CD11b+CD103+ DCs within the LP was increased, while *B. infantis* feeding did not influence CD11b-CD103+ DC population. CD11b+CD103+ DC population was shown to play a key role in Th17 cell differentiation *in vitro* (281). However, their role *in vivo* was not fully established. In our model the connection between *B. infantis* associated CD11b+CD103+ DCs suppression and Th17 decrease in the gut were clearly seen. Recently IRF4 was identified as a critical regulator of this DC population efficient in Th17 cells priming (284). All DSS-treated groups had elevated numbers of CD103+ DCs but further characterization was critical. *B. infantis* administration induced regulatory CD11b-CD103+ RALDH active DCs within the LP and suppressed the increase in CD11b+CD103+ DCs. These CD11b-CD103+ RALDH+ DCs were involved in retinoic acid dependent suppression of inflammation. Taking this data together CD103+ DCs may be possibly divided into two differently behaving groups: CD11b+CD103+ DCs with strong pro-inflammatory properties and CD11b-CD103+ DCs with anti-inflammatory properties, but this requires further investigation.

8.6. *B. infantis*-induced suppression of Th1 and Th17 cells in the gut

As the role for retinoic acid in Foxp3 Treg induction is well described, this lymphocyte population was analyzed in LP and MLN. Foxp3 Treg cells were increased in LP of healthy mice, however this effect was retinoic acid-independent. However, IL-10 producing Tr1 lymphocytes were increased in MLN in a retinoic acid-dependent manner. This suggests a role for other factors in immunoregulatory effects driven by *B. infantis.* The synergistic role of retinoic acid and TGF-β is well described (215, 247). There is potential for a TGF-β effect on Treg cells in LP, partially supported by the observation that MDDCs stimulated with *B. infantis* were able to secrete TGF-β. (data unpublished) *B. infantis* oral administration also had effects on other CD4+ T cell subsets. Th1 and Th17 cells were decreased in a retinoic acid-dependent manner in healthy and inflamed LP. These effects on Th17 cells may be mediated indirectly as the Th17 cell priming environment was influenced by attenuation of IL-1β and IL-6 secretion. Production of these cytokines by LP DCs could be potentially reduced via SOCS3 induction, as it was previously mentioned in the discussion. Th1 and Th17 populations may be reduced by Treg cells, which can directly act on pro-inflammatory T lymphocytes or indirectly (via DCs) reduce inflammatory T subsets priming. CTLA-4+ Tregs seems to be the most efficient for contact dependent regulation of CD80 and CD86 expression. Similarly, lymphocyte-activation gene 3 (LAG3) may block DC maturation. Treg cells secrete immunosuppressive cytokines, such as IL-10 and TGF-β and during division consume IL-2 required for efficient T cell proliferation. Galectin-1 and granzyme B production and release induce effector T cell apoptosis (285). Interestingly, Tregs express CD39 and CD73 ectoenzymes generating adenosine from pericellular ATP. Adenosine binding to $A_{2A}R$ receptor leads to inhibition of T effector cells. Moreover, adenosine inhibits IL-6 production, while promoting TGF-β and Treg generation (286). As human pDCs were shown to produce IDO in response to *B. infantis* there is a possibility that also murine pDCs can react in similar manner. IDO promotes Treg development and leads to tryptophan starvation and tryptophan metabolite accumulation, which result in subsequent inhibition of T cell effector functions.

8.7. Regulation of gut homing receptor expression by retinoic acid and other factors

Retinoic acid was shown to induce gut homing receptors on activated B and T lymphocytes (185, 213, 230). However, *B. infantis* administration in healthy animals led to decrease in α4β7 and CCR9 expression on T cells within LP and for CCR9 this effect was retinoic acid dependent. Retinoic acid-induced CCR9 expression can be reduced in the presence of TGF-β (287), which upregulates the β_7 subunit and CD103 (288). This can result in low level of CCR9 and high level of α4β7 on Treg cells. α4β7 Tregs can home not only to small intestine, but also to the colon and other mucosal sites, such as the respiratory tract and sites of inflammation, where the α4β7 ligand MadCAM is highly expressed. This can potentially explain why *B. infantis* administration led to the decrease in α4β7 CD4+ T lymphocytes. In contrast, retinoic acid-dependent reduced CCR9 expression can be due to a reduction in Th17 and Th1 cells in the small intestine lamina propria, as Th17 lymphocytes were shown to express both gut homing receptors α4β7 and CCR9 at low concentrations of retinoic acid (287). Moreover, during DSS-induced colitis no effect on gut homing receptors expression on CD4+ T cells was observed. However, it is well known that CCR9 deficiency in mice exacerbates colitis and Treg cells gut homing is impaired (289, 290). This suggests that lack of gut homing receptor expression changes during colitis after *B. infantis* administration can be due to strong gut homing of pro- and anti-inflammatory CD4+ T cells together to the small and large intestine.

8.8. Vitamin A and other dietary factors interacting with microbiota and immune system

Retinoic acid metabolism seems to be critical for protection against DSS-induced colitis. *B. infantis* has protective effects in IL-$10^{-/-}$ mice (30), which suggests that other factors induced by this bacterium are important. In healthy and inflamed LP Th1 and Th17 subsets are decreased in a retinoic acid-dependent manner. Moreover, myeloperoxidases activity and histological score are decreased only in mice fed with *B. infantis*. This effect is again retinoic acid- dependent as citral (RALDH enzymes inhibitor) suppressed the attenuation of inflammation.

The induction of retinoic acid metabolism by *B. infantis* suggests a link between microbiota, diet, metabolism and immunoregulation. These results suggest that vitamin A and *B. infantis* can act synergistically and together contribute to improving immune homeostasis. There is growing evidence for the interaction between diet components, microbial metabolites and the immune system. Nondigestible carbohydrates may influence bacterial growth and are transformed by microbes into new polysaccharide structures regulating immune responses. Moreover, SCFA are produced by *Bifidobacteria* and *Lactobacilli* strains during fiber fermentation and contribute to immunoregulation (47). However, there are more microbial metabolites, such as long-chain fatty acids (LCFAs) and biogenic amines, produced from dietary components within the gut. Sugar, antioxidants, certain short-chain carbohydrates and sodium salts of SCFA promote conjugated linoleic acids (CLAs), belonging to LCFAs, production by *Bifidobacteria* in the small intestine (291, 292). In addition, biogenic amines, such as histamine and γ-amino butyric acid (GABA), are produced by bacteria from accessible amino acids or from aldehydes and ketones. This suggests that specially formulated diets composed of microbes and dietary components can enhance immunoregulatory responses. In addition, precise monitoring of dietary habits during commensal microbes intervention studies may identify subgroups that respond better to therapy.

8.9. Microbial compounds as new therapeutics

It is unknown which component(s) of *B. infantis* is responsible for its immunoregulatory effects. Potentially it could be an exopolysaccharide, which is produced in high amounts by this bacterium. Until now polysaccharide A from *Bacteroides* was described as an immunoregulatory polysaccharide, which acted via TLR2 (98). However, there is also growing evidence suggesting that bacterial proteins are immunoregulatory, for instance in the induction of skin homing receptors (293). Isolating specific bacterial compounds and using them instead of the intact bacteria can be a promising direction for future therapeutic uses. It allows for application not limited to the gut and for new forms of administration. Albeit, SNPs in PRRs, which predispose to certain diseases can became a critical limitation in this type of therapy. Mutation in the PRR may block ligand binding or lead to differential signaling pathway activation via the altered receptor. For instance, R753Q polymorphism in TLR2 increases tuberculosis incidence via impaired dimerization with TLR6 and recruitment of MyD88 (294). The same mutation may interfere with a bacterial compound acting via heterodimer TLR2/6 in order to induce tolerogenic responses.

8.10. Conclusion and outlook

The microbiota in the gut lumen is continuously monitored by the GALT. Microbes are sampled by LP DC intra-epithelial dendrites or are delivered to PP resident DCs through M cells. Bacterial components are recognized by diverse PRRs, which are present on the surface, in the intracellular compartments and in the cytoplasm of DCs. Moreover, DCs respond to microbial metabolites produced in the gut lumen during fermentation processes. Bacterial activated DCs secrete diverse cytokines, immunomodulatory factors and interact with T and B lymphocytes, which leads to pro- or anti-inflammatory adaptive responses. Antigen presentation takes place locally in LP and after DC migration to the MLN. In addition, some DCs can stay within PP and prime naïve T cells.

All these processes are involved in tolerance induction to non-pathogenic gut microbiota and in case of infection by enterogenic microbes provide pro-inflammatory responses and support pathogen eradication. However, sometimes the immune system responds to commensals in an inappropriate manner resulting in chronic inflammation. This can be due to disregulation on the DC level, including impaired recognition by mutated PRRs, secreting pro-inflammatory factors in response to commensal microbes and creating an environment, which supports Th1 and Th17 cell generation. Another possibility is related to regulatory mechanisms, such as Treg functionality, impairment and prolonged mucosal inflammation after infections. Moreover, dietary habits and antibiotics can modulate the gut microbiome and cause changes in quality and quantity of commensal microbes. This may favor microbes promoting mucosal inflammation. However, it remains unknown if changes in microbiota lead to inflammatory disorders or if they are a consequence of local inflammatory responses.

An appropriate diet combined with specific commensal microbes administration could be a potential treatment of intestinal inflammation. Induction of tolerogenic signals and metabolites, such as retinoic acid may help to restore immune homeostasis in the gut, by improving anti-inflammatory innate and adaptive responses. *B. infatis* serves as very good example of a tolerogenic microbe preventing and reducing inflammation within the gastrointestinal tract. In this thesis we described molecular mechanisms leading to tolerance induction by this bacterium. *B. infatis* is sampled from

the gut lumen by DCs and enhances vitamin A metabolism in LP CD103+ DCs, leading to subsequent Foxp3+ Tregs generation (Figure 10). Moreover, pro-inflammatory Th1 and Th17 populations are decreased within the LP. Human mDCs recognize *B. infatis* via TLR2 and DC-SIGN, while pDCS sense this bacterium via TLR9. Bacterial activated DCs produce IL-10, TGF-β, IDO and RALDH2, which leads to Tregs generation.

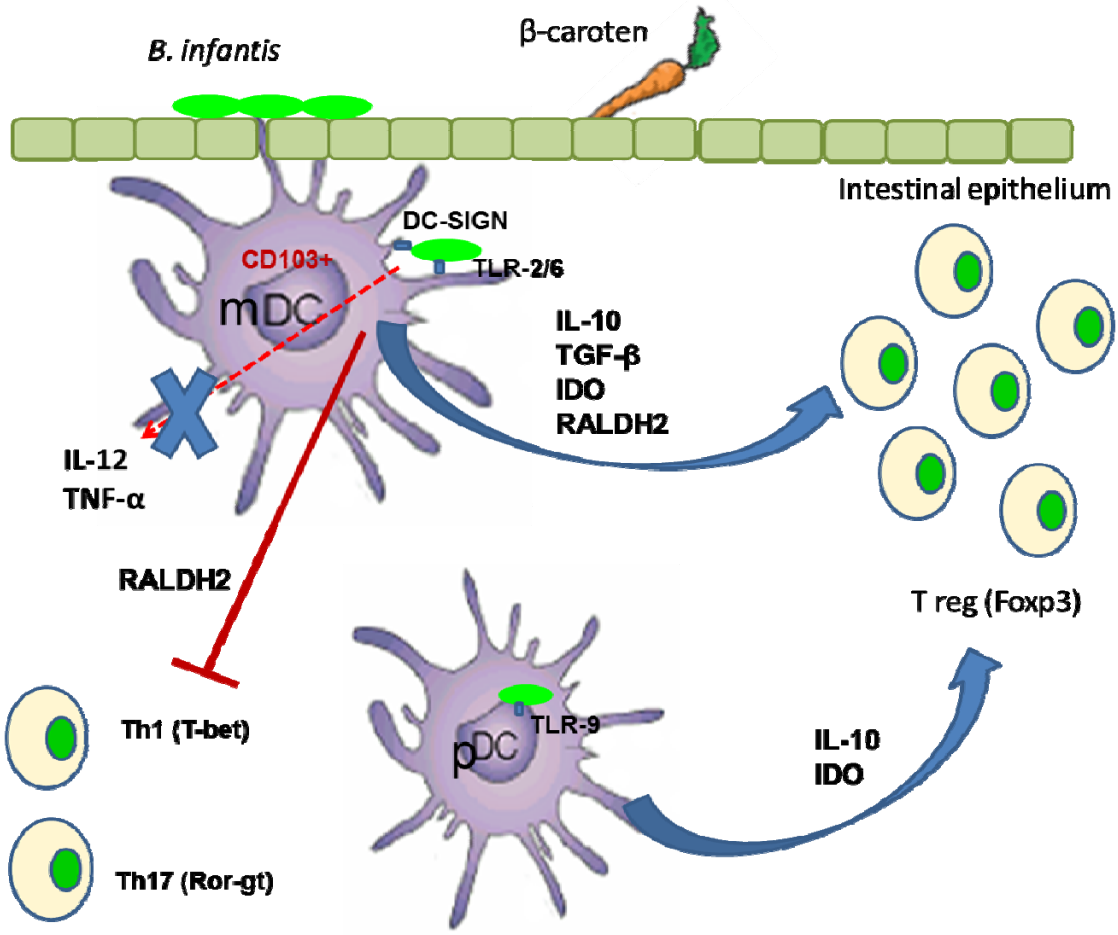

Figure 10. *B. infatis* immunoregulatory effects.

B. infatis administration together with vitamin A may be beneficial for patients suffering from diverse intestinal inflammatory disorders. Moreover, recent reports about the positive impact on non-gut related diseases, such as psoriasis, opens the possibility for specific supplementation in other inflammatory conditions. Interestingly, the development of immunoregulatory bacterial compounds may broaden the spectrum of applications and could lead to new medicaments.

8.11. Contribution statement

In the article "*Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells" experiments for Figures 1-3 were performed by David Groeger. Results for Figure S1 were prepared by Mario Ziegler and Liam O'Mahony. I performed other experiments (Figures 4-7 and Figures S2-S6) with help from Remo Frei, Ruth Ferstl and Mario Ziegler.

For the article "Portrait of an immunoregulatory Bifidobacterium" I performed experiments presented in Figure 2.

For the article "Immunomodulation by *Bifidobacterium infantis* 35624 in the murine lamina propria requires retinoic acid-dependent and independent mechanisms" work with live animals was done by Ruth Ferstl. Swiss role sections for Figure 5 were analyzed by Dirk Nehrbass. I performed all experiments with help from Ruth Ferstl, Remo Frei and Mario Ziegler.

All experiments were supervised by Liam O'Mahony.

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10. Curriculum Vitae

PERSONAL INFORMATION:

Family name:	Konieczna
First name:	Patrycja
Middle name:	Anna
Address:	Promenade 112 Davos Platz 7270 Switzerland
Date of birth:	28.11.1985
Place of birth:	Poznań
Nationality:	Polish

EDUCATION:

2009-2013	PhD in molecular immunology, Swiss Institute of Allergy and Asthma Research (SIAF) in Davos, University of Zurich, Switzerland PhD thesis: **The molecular mechanisms mediating dendritic cell responses to anti-inflammatory microbes.**
07.01.-07.07.2013	Alimentary Health Ltd. in Cork, Ireland TEAM-EPIC Training Network Bacterial exopolysaccharides isolation and purification
2004 - 2009	Master of Science, Poznań University of Life Sciences (Poland), faculty - biotechnology, specialization – industrial biotechnology; M.Sc.Eng., Title of the thesis: **Analysis of probiotic yeasts protein-protein interactions in Yeast Two-Hybrid System (ProQuest, INVITROGEN)**

COURSES:

Training School, COST Action BM0806
Nafsika Palace, Itea, Greece (26 – 28 July 2012)
Methodological Strategies for Histamine Receptor Characterization

Microscopy Winterschool 2012
Zurich, Switzerland (15 – 20 January 2012)
Practical Course in advanced 3D Microscopy

Presentation Course
Zurich, Switzerland (15/24 June 2011 and 13 July 2011)
Presenting Science

LTK Module 1E: Introductory Course in Laboratory Animal Science
Zurich, Switzerland (24 January – 2 February 2011)

Immunology Lectures
Davos, Switzerland
(Fall semester 2010 – Spring semester 2011)

4th MIM Introductory Course
Zurich, Switzerland (7 – 11 June 2010)

Cell Sorting Course
Davos, Switzerland (11 – 12 March 2010)

JOURNAL CLUBS AT SIAF:

19 OCTOBER 2010

08 MARCH 2011

01 NOVEMBER 2011

03 JULY 2012

29 OCTOBER 2012

PROGRESS REPORTS AT SIAF:

16 JULY 2010

23 NOVEMBER 2010

05 JULY 2011

01 DECEMBER 2011

19 JULY 2012

06 NOVEMBER 2012

COMMITTEE MEETINGS:

13.12.2010 "THE MOLECULAR BASIS FOR INDUCTION OF REGULATORY DENDRITIC CELLS BY THE COMMENSAL MICROBIOTA"

01.03.2012 "MICROBIOME AND IMMUNITY REGULATION"

10.12.2012 "RELATIONSHIP BETWEEN MICROBES, HISTAMINE AND RETINOIC ACID"

09.09.2013 "EPS ISOLATION – SIMPLE, EFFICIENT AND PURE"

CONFERENCES:

WIRM VII World Immune Regulation Meeting-VII

"Innate and Adaptive Immune Response and Role of Tissues in Immune Regulation"

Davos, Switzerland (13-16 March 2013)

***Bifidobacterium infantis*-induced CD103+RALDH+ Dendritic Cells Protect Against DSS Colitis**

Konieczna Patrycja, Ferstl Ruth, Ziegler Mario, Frei Remo, O'Mahony Liam

Oral presentation

Grabünden forscht- Young Scientists in Contest

Davos Congress Centre, Switzerland (12-13 September 2012)

***Bifidobacterium infantis* activated plasmacytoid dendritic cells induce regulatory T cells**

Konieczna Patrycja, Ziegler Mario, Frei Remo, Ferstl Ruth, O'Mahony Liam

poster

EAACI Congress 2012 Geneva, Switzerland (16-20 June 2012)

***Bifidobacterium infantis* activated plasmacytoid dendritic cells induce regulatory T cells**

Konieczna Patrycja, Ziegler Mario, Frei Remo, Ferstl Ruth, O'Mahony Liam

Poster discussion session

24th Meeting of the Swiss Immunology PhD students, Wolfsberg meeting (2-4 April 2012)

Plasmacytoid dendritic cells induce Foxp3 regulatory T cells in response to *Bifidobacterium infantis*

Konieczna Patrycja, Ziegler Mario, Frei Remo, Ferstl Ruth, O'Mahony Liam

Oral presentation

WIRM-VI World Immune Regulation Meeting -VI
"Innate and Adaptive Immune Response and Role of Tissues in ImmuneRegulation"
Davos, Switzerland (18 - 21 March 2012)
The Role of Plasmacytoid Denedritic Cells in Foxp3 regulatory T cells induction by commensal microbiota
Konieczna Patrycja, Ziegler Mario, Frei Remo, Ferstl Ruth, O'Mahony Liam
Poster presentation

23rd Meeting of the Swiss Immunology PhD students
Wolfsberg meeting (30 March – 1 April 2011)
The Molecular Basis for Induction of Regulatory Dendritic Cells by the Commensal Microbiota
Patrycja Konieczna, Mario Ziegler, Remo Frei, Ruth Ferstl, Cezmi Akdis, Liam O'Mahony
Poster presentation

WIRM-V World Immune Regulation Meeting -V
"Innate and Adaptive Immune Response and Role of Tissues in Immune Regulation"
Davos, Switzerland (24 - 27 March 2011)
The Molecular Basis for Induction of Regulatory Dendritic Cells by the Commensal Microbiota
Patrycja Konieczna, Mario Ziegler, Remo Frei, Ruth Ferstl, Cezmi Akdis, Liam O'Mahony
Poster presentation

EAACI Winter School,
Davos, Switzerland, (3-6 February 2011)
The Molecular Basis for Induction of Regulatory Dendritic Cells by the Commensal Microbiota
Patrycja Konieczna, Mario Ziegler, Ruth Ferstl, Liam O'Mahony
Oral presentation

ARTICLES:

Tordesillas L, Gómez-Casado C, Garrido-Arandia M, Murua-Garcia A, Palacin A, Varela J, **Konieczna P**, Akdis C, O'Mahony L, Diaz-Perales A. Transport of Pru p 3 across gastrointestinal epithelium - An essential step towards the induction of food allergy? (2013) **Clin Exp Allergy** submitted

Konieczna P, Ferstl R, Ziegler M, Frei R, Nehrbass D, Lauener RP, Akdis CA, O'Mahony L. Immunomodulation by *Bifidobacterium infantis* 35624 in the murine lamina propria requires retinoic acid-dependent and independent mechanisms. (2013) **PLoS One** 8:e62617.

Frei R, Ferstl R, **Konieczna P**, Ziegler M, Simon T, Rugeles TM, Mailand S, Watanabe T, Lauener R, Akdis CA, O'Mahony L. Histamine receptor 2 modifies dendritic cell responses to microbial ligands. (2013) **J Allergy Clin Immunol** 132:194-204.e12.

Konieczna P, Akdis CA, Quigley EM, Shanahan F, O'Mahony L. Portrait of an immunoregulatory Bifidobacterium. (2012) **Gut Microbes** 3:261-6.

Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, Quigley EM, Kiely B, Akdis CA, O'Mahony L. *Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. (2012) **Gut** 61:354-66.

O'Mahony D, Murphy S, Boileau T, Park J, O'Brien F, Groeger D, **Konieczna P**, Ziegler M, Scully P, Shanahan F, Kiely B, O'Mahony L. *Bifidobacterium animalis* AHC7 protects against pathogen-induced NF-κB activation in vivo. (2010) **BMC Immunol** 11:63.